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State Board of Health, Indianapolis	January 29, 1949
PACIFIC COAST	
Stanford University	April 9, 1949
SOUTHERN	
Tulane University	April 8, 1949
SOUTHERN CALIFORNIA	
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WESTERN NEW YORK	
University of Rochester	February 19, 1949

17058. A Comparative Study of the Local Toxic Action of Mercurial Diuretics.

Robert A. Lehman, Harry Taube, and E. E. King.
From Department of Therapeutics, New York University College of Medicine, New York City.

The mercurial diuretics have today a secure place in the management of the edematous patient because of their effectiveness and dependability. However they give rise, on occasion, to various side effects of which irritation at the site of injection and an acute toxic action on the heart are the most significant. This latter action may sometimes be fatal[1-3] and in an attempt to eliminate it a new type of mercurial has recently been made available under the name Thiomerin.* This drug is identical with Mercuzanthin (Mercurophylline, USP XIII) except that it contains sodium mercapto acetate in chemical combination with the mercury instead of theophylline. In animal experiments it failed to produce the typical ventricular tachycardia and fibrillation which occurs after the other diuretics.[4] Thiomerin also appears to be less toxic to tissue and has been proposed for clinical use by subcutaneous injection. It therefore seemed desirable to make a detailed comparison of the mercurial diuretics with respect to the reaction which they produce at the site of injection. Thiomerin, Mercuzanthin, and Mercuhydrin were used in this study.

Subcutaneous Injection in Mice. It has

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[1] DeGraff, A. C., and Nadler, J. E., *J. Am. Med. Assn.*, 1942, **119**, 1006.

[2] Ben-Asher, Solomon, *Ann. Int. Med.*, 1946, **25**, 711.

[3] Volini, Italo, Levitt, R. O., and Martin, Richard, *J. Am. Med. Assn.*, 1945, **128**, 12.

* Thiomerin has also been identified as MT6.

[4] Lehman, R. A., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 428.

previously been shown in rabbits, that addition of theophylline to a parent mercurial such as Salyrgan or Mercuzan, protects the skin from the severe necrosis following intradermal injection.[5] However, this technic was not found to give a sufficiently sensitive or consistent response for the study of mercurials of a less irritant character. The albino mouse was selected as a more suitable animal since, unlike the rabbit, the skin over the abdomen is thin enough so that necrosis cannot escape detection, and large enough groups of animals can be used to evaluate the effect of individual variation.

Three groups of 5 mice in the weight range of 20 to 30 g were selected at random and the hair carefully clipped over the abdomen. The first group received Thiomerin, the second Mercuzanthin and the third Mercuhydrin all by subcutaneous injection in a dose of 0.03 cc of undiluted solution as supplied for clinical use (40 mg of mercury per cc). A one quarter cc tuberculin syringe with a 27 gauge hypodermic needle was used with the bevel up. All the animals were injected on the same day. Approximately 24 hours after the injection the mice were examined for evidences of toxicity. They were then anesthetized with a barbiturate and photographed in color. These photographs appear as Fig. 1.

It will be observed that in the case of Mercuzanthin the destruction of tissue has progressed in all animals to the point of skin breakdown over a substantial area of the abdomen. The very dark discoloration accompanying some of the lesions suggests the presence of extravasated blood. In every case there is a white ring surrounding the area of necrosis which is presumably due to inflammatory exudate.

The response to Mercuhydrin is of similar character but somewhat less severe, more superficial, and involves a smaller area of skin. Nevertheless perforation of the skin will be observed in the first animal on the left in Fig. 1. Extravasation of blood appears not to have occurred in this group.

None of the animals injected with Thiomerin shows pathology which can be detected on gross examination.

Intramuscular Injection in Rats. Because of these marked differences in local toxicity, studies were undertaken to compare the histopathology occurring after injection of Thiomerin, Mercuzanthin and Mercuhydrin. For this purpose the tibialis anterior muscle of the rat was selected as a more homogeneous tissue than the skin for the precise administration of a measured volume of drug. Each rat was etherized very lightly, the hair shaved from the leg and 0.03 cc of undiluted drug was injected into the belly of the muscle using a one-quarter cc syringe and 27 gauge needle. Twenty of the rats received saline control injections into the corresponding muscle of the opposite leg. The rats were killed by a blow on the head after a period of 4, 24, or 96 hours. The skin was stripped away and the injected muscle gently freed from adherent muscle bundles and dissected free. Muscles were fixed in Bouin's solution, embedded in paraffin and cut at 10 μ. Each muscle was sectioned serially at intervals of 500 μ. The sections were examined by the pathologist without foreknowledge of the treatments given and the pathology was scored as "none", "slight", "moderate", "marked", or "very marked" in the following categories: "disruption of muscle integrity", "cellular infiltration", and "edema". The results are given in the Table.

Disruption of Muscle Integrity. It appears from the Table that after 4 or 24 hours the 3 drugs show definite disruption of tissue in distinction to the saline control. After 96 hours the disruption is less than at 4 or 24 hours in the case of Mercuzanthin and Mercuhydrin while Thiomerin injected muscles now show no disruption and cannot be distinguished from the controls.

Cellular Infiltration. After 4 hours all of the drugs as well as saline have produced a slight cellular response as indicated in the Table. Twenty-four hours after injection the response to saline has not increased while that to the three mercurials has increased a great deal. After 96 hours the infiltration has disappeared in the case of Thiomerin and

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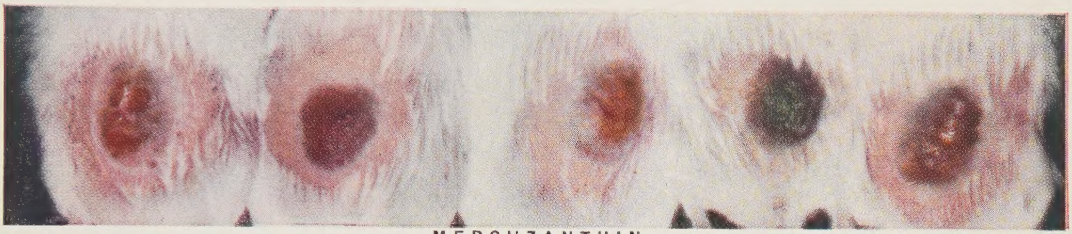
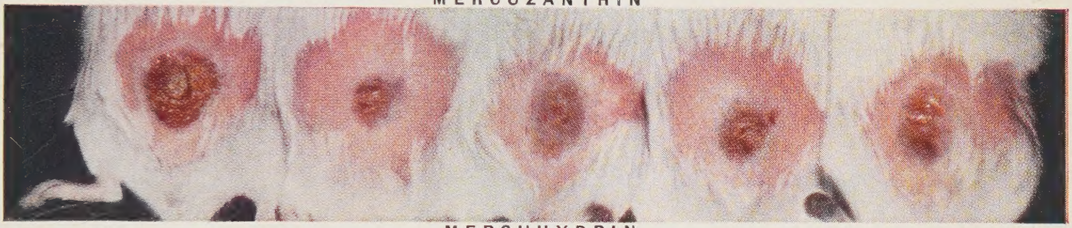
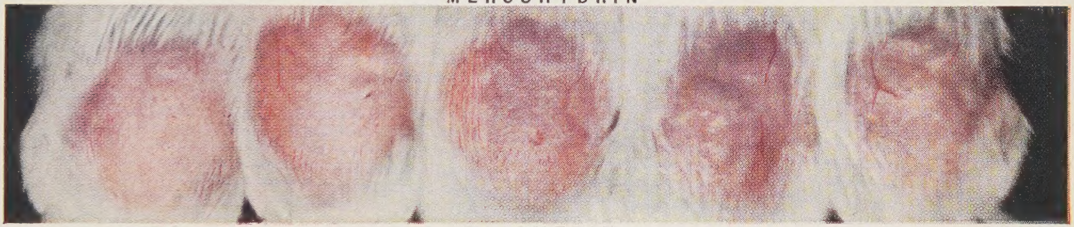
[5] DeGraff, A. C., Batterman, R. C., and Lehman, R. A., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 373.



MERCUZANTHIN



MERCUHYDRIN



THIOMERIN

Fig. 1

Photographs of mice 24 hours after the subcutaneous injection of 0.03 cc. of a mercurial diuretic at a concentration of approximately 40 mgm of mercury per cc. All of the mice in a row received the same treatment. *Top Row*: Mercuzanthin (Mercurophylline USP XIII); *Middle Row*: Mercuhydrin (Meralluride); *Bottom Row*: Thiomerin.

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TABLE I.
Scores for Examination of Muscle Sections After Injection of Mercurial Diuretics.*

	4 hr			24 hr			96 hr		
Drug	DI	CI	ED	DI	CI	ED	DI	CI	ED
Saline	0	+	0	0	0	0	0	0	0
	0	+	+	0	+	0	0	0	0
	0	+	+	0	+	0	0	0	0
	0	+	+	0	+	0	0	+	0
	+		+	0	+	0	++	+++	+
				0	+	+			
				0	+	+			
				0	+	+			
				+	+	+			
				+	++	+			
Thiomerin	+	+	+	+	++	+	0	0	0
	+	+	++	+	++	++	0	0	0
	+	+	++	++	++	++	0	+	0
	++	++	++	++	++	++	0	+	0
	++	++	++	++	++	++	0	+	+
				++	+++	++			
				++	+++	++			
				++	+++	++			
				++	+++	++			
					+++	+++			
Mercuzanthin	+	+	+	++	++	+	0	++	+
	+	+	+	++	+++	++	+	++	+
	++	+	+	+++	+++	++	++	++	+
	++	+	++	+++	++++	++	++	++	++
				+++	++++	+++	++	+++	++
Mercuhydrin	+	+	+	+	++	+	+	++	+
	++	+	+	++	++	++	++	+++	+
	++	+	++	++	++	++	++	+++	+
	+++	+	++	+++	+++	++	++	+++	+
	+++		++	+++	+++	++			

* 0 none; + slight; ++ moderate; +++ marked; ++++ very marked.
DI, disruption of muscle integrity; CI, cellular infiltration; ED, edema.

saline but is still present in the case of Mercuzanthin and Mercuhydrin.

Edema. Four hours after injection edema is present in every instance although saline shows less than the 3 drugs. The edema persists for at least 24 hours. After 96 hours it has disappeared from the saline and Thiomerin injected muscles but is still present in the others. It should be pointed out in this connection that absorption of these drugs is substantially complete one hour after injection.[6]

The character of the response is illustrated in Fig. 2, 3, and 4. Fig. 2 is typical of the response 24 hours after injection of any one of the 3 mercurials and is characterized by the infiltration of polymorphs, lymphocytes and the appearance of edema fluid. The response to saline was similar but quantitatively less extensive. Fig. 3 illustrates the appearance of muscle 96 hours after injection of Mercuzanthin or Mercuhydrin and shows not only the persistence of the inflammatory exudate suggesting continued irritation but also the development of a reparative response as manifested by newly-formed connective tissue. Fig. 4 shows essentially normal muscle which was consistently observed 96 hours after injection of Thiomerin or saline.

Discussion. It has long been taken for granted that mercury compounds in which at least one valence bond bears an ionizable group are highly irritant to tissues. Thus Salyrgan with the configuration $\mathrm{R}\overset{+}{\mathrm{Hg}}—\overset{-}{\mathrm{OH}}$ is severely and rapidly necrotizing.[5] The introduction of Mercuzanthin provided a compound of structure RHg—N< in which the irritant action of the mercury has been considerably reduced by combination with theo-

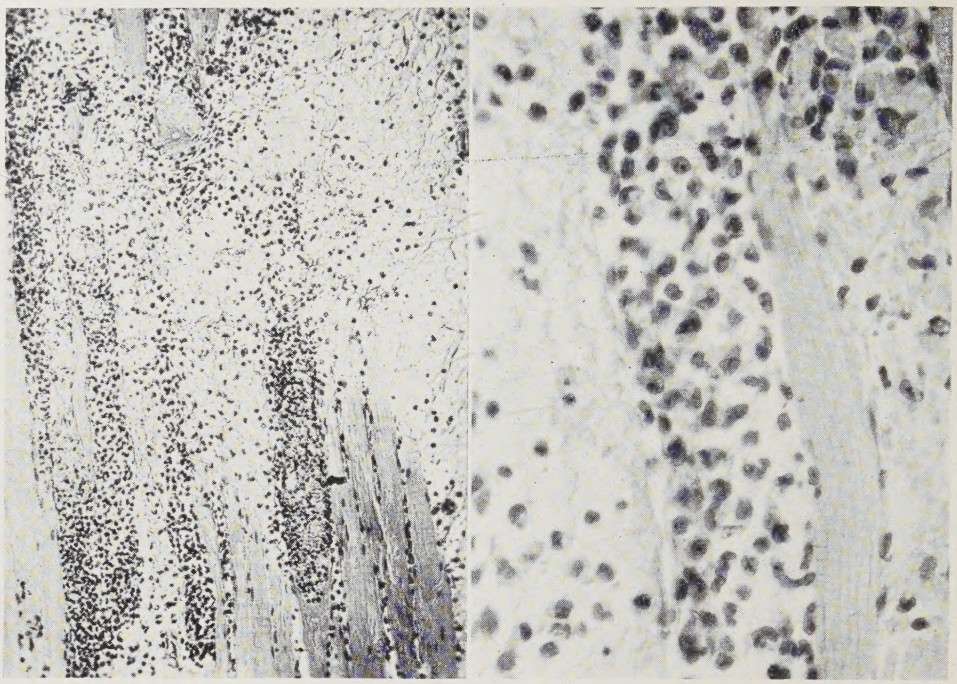

FIG. 2.

Section of rat muscle illustrating the response to Mercuzanthin, Mercuhydrin or Thiomerin 24 hr after inj. Left—×100; right—×450.

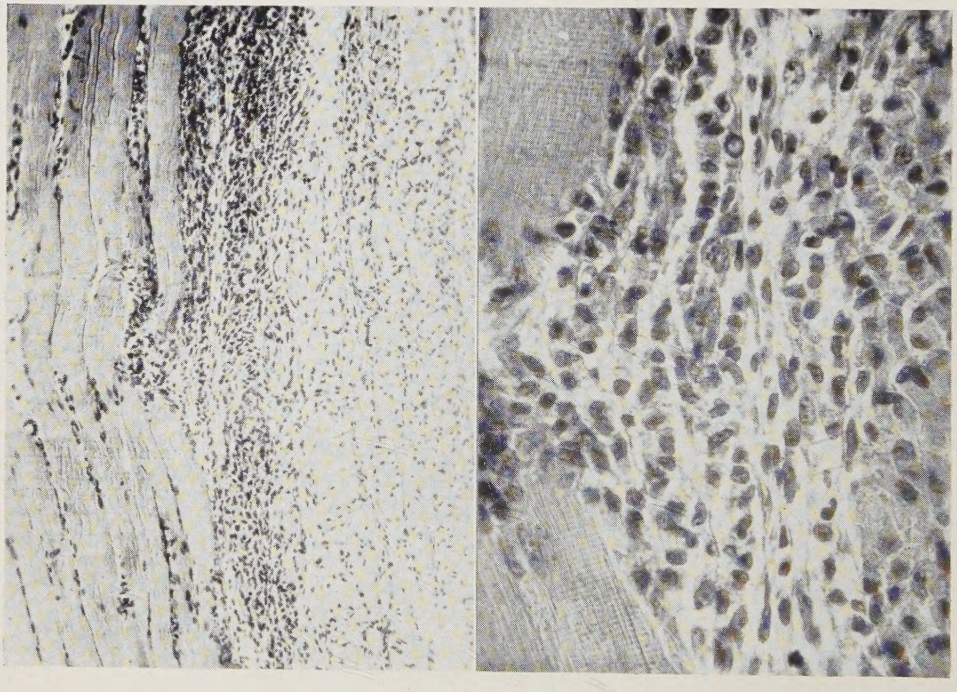

FIG. 3.

Section of rat muscle illustrating the response to Mercuzanthin or Mercuhydrin 96 hr after inj. Left—×100; right—×450.

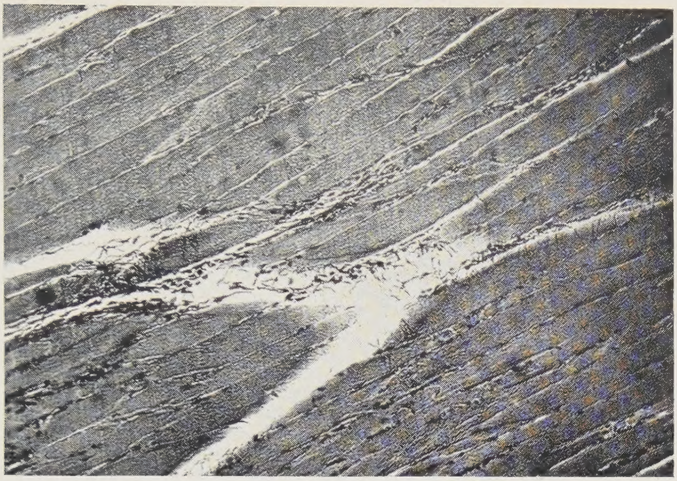

FIG. 4.
Section of rat muscle 96 hr after inj. of Thiomerin or saline showing essentially normal tissue. ×100.

phylline. Nitrogen compounds of similar structure (such as succinimide) can be substituted for theophylline with comparable effect.[6,7] Salyrgan-theophylline and Mercuhydrin are theophylline-bearing drugs of this class. In Thiomerin the second valence bond of the mercury has been bound as a mercaptide to give a substance of the formula RHg–S– and this modification has been shown above to result in a drug of still less toxicity. In a previous report it was indicated that the addition of a sulfhydryl compound to a conventional diuretic will cause precipitation of the theophylline indicating that the Hg–S bond is chemically more stable than the Hg–N.[4] Hence the results here reported would be consistent with the conclusion that local irritant action in the mercurial diuretic series is proportional to the reactivity of the mercury which they contain. A logical extension of this series would be the compound RHg—C <- which might be expected to show even less local irritant action than Thiomerin. However, such a mercurial would also be expected not to have therapeutic activity.

It is beyond the scope of this report to discuss the effect of the Hg–S bond on diuretic activity. Clinical studies[8–10] seem to indicate that Thiomerin is at least as effective as the other mercurials in this respect.

It may be mentioned in passing that the sensitivity of mice to the subcutaneous injection of mercurials was found to be greater in the summer than in the winter months. All of the injections for the experiment which is illustrated in Fig. 1 were accordingly given on the same day (September) so that the comparison would be valid. In this connection the work of DiPalma and coworkers[11] on seasonal variations in the sensitivity of the skin to the production of reactive hyperemia is of interest.

Summary and Conclusions. Three mercurial diuretics, Thiomerin, Mercuzanthin and Mercuhydrin have been compared with respect to the tissue reaction which they produce. Thiomerin was tolerated by mice on subcutaneous injection without exhibiting gross pathology while Mercuzanthin and Mercuhydrin gave

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[6] Lehman, R. A., and Dater, Arnold, *J. Pharm. Exp. Therap.*, 1938, **63**, 443.

[7] Hunt, W. H., Walter, L. A., and Fosbinder, R. J., *J. Am. Pharm. Assn.*, Sci. ed., 1942, **31**, 278.

[8] Herrmann, George R., Chriss, John W., Hejmancik, Milton R., and Sims, Paul M., *Texas State J. Med.*, 1949, **45**, 79.

[9] Grossman, J., Weston, R. E., Edelman, I. S., and Leiter, L., in press.

[10] Batterman, Robert C., Unterman, David, and DeGraff, Arthur C., *J. Am. Med. Assn.*, in press.

[11] DiPalma, J. R., Reynolds, S. M. R., and Foster, F. I., *Am. Heart J.*, 1942, **23**, 377.

rise to necrosis of the skin under the same conditions. After intramuscular injection in rats all three drugs gave an early inflammatory response characterized by the appearance of a polymorphonuclear exudate. In the case of Thiomerin this exudate was entirely resorbed without evidence of residual damage. After injection of Mercuzanthin, or Mercuhydrin, however, the irreversible nature of the response was indicated by marked fibroblastic proliferation.

These results would seem to furnish an adequate experimental basis for the clinical use of Thiomerin by subcutaneous injection.

The authors wish to thank the Misses Florence Katine and Josephine Brosseau for able technical assistance.

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17059. Observations of Nitrite-Induced Postural Syncope in Patients with Mental Disease.

M. D. Altschule and M. Lorenz.
From the Laboratory of Clinical Physiology, McLean Hospital, Waverley, Mass., and the Department of Medicine, Harvard Medical School, Boston, Mass.

Earlier work[1] discussed the vasomotor system in schizophrenia; it was concluded that no structural vascular defect exists and that schizophrenic patients often exhibit acral vasoconstriction. Investigation of the reactivity of postural vasoconstrictor mechanisms has also been made. Nitrite dilates vessels by relaxing smooth muscle, but does not prevent the action of nervous impulses on this muscle; accordingly, the degree to which the vasoconstriction overcomes the effect of nitrite affords data on the reactivity of postural vasoconstrictor mechanisms.

Material and methods. Twenty-four patients were studied. Their diagnoses varied (Table I). Four (Table II), all schizophrenics were studied only after lobotomy. Twenty-four normal subjects were also observed. After reassurance the subjects came to the laboratory fasting or 3 or 4 hours after a last meal. Patients were not permitted to smoke before or during experiments. Each subject was given 3 grains of sodium nitrite by mouth and allowed to rest for 30 minutes. He then lay on a board which had no protuberances except for a bicycle seat at right angles to it; this the patient straddled. After control observations were made, the board was tilted 60 degrees leaving the patient head-up with legs hanging. Systolic blood pressure was measured every few minutes by palpation. Auscultatory measurements were not made since they may be markedly in error with changing vasomotor tone; an arterial cannula was considered undesirable for measuring arterial pressure in this study. If syncope did not occur within forty minutes after tilting the patient, the observations were ended and were repeated no sooner than 2 days later, using 7 grains of sodium nitrite.

Observations. Subjects were classified as follows:

Group I. Syncope during tilting after 3 grains of sodium nitrite, *i.e.*, no increase in vasomotor reactivity (Fig. 1).

Group II. Syncope during tilting after 7 grains but not after 3 grains of sodium nitrite, *i.e.*, possible increase in vasomotor reactivity (Fig. 2).

Group III. Fall in blood pressure during tilting but no syncope after 7 grains of sodium nitrite, *i.e.*, definite increase in vasomotor reactivity (Fig. 3).

Group IV. Rise in blood pressure during tilting after 7 grains of sodium nitrite, *i.e.*, marked increase in vasomotor reactivity (Fig. 4).

Twenty-two normal subjects were in Group I and 2 in Group II. Seven of the 11 non-lobotomized schizophrenic patients exhibited

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[1] Altschule, M. D., and Sulzbach, W. M., *Arch. Neurol. and Psychiat.*, 1949, **61**, 44.

TABLE I.
Vasomotor Response to Tilting After Administration of Nitrite.

Diagnosis	No. of cases				
	Groups I	II	III	IV	Total
Normal	22	2	—	—	24
Schizophrenia	3	1	5	2	11
Organic disease—post asphyxial	—	—	1	—	1
Involutional psychosis, paranoia	1	—	—	—	1
Manic-depressive, manic	—	1	—	—	1
Anxiety neurosis	—	—	1	—	1
Psychoneurosis, reactive depression	1	—	1	—	2
Chronic alcoholism	—	2	1	—	3
Total, non-schizophrenic	2	3	4	0	9

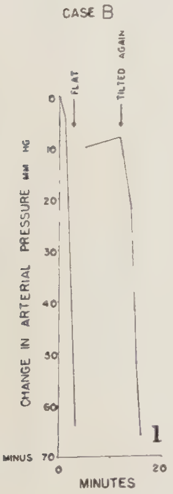
TABLE II.
Vasomotor Response to Tilting After Administration of Nitrite in Lobotomized Patients.

Case	Group		Diagnosis
	Before	After	
We	III	I	Schizophrenia, paranoid
Wi	II	III	,, ,,
B	—	I	,, catatonic
S	—	III	,, ,,
L	—	IV	,, ,,
Wo	—	IV	Schizophrenia, other types
Z	I	I	,, ,, ,,

a definite or marked increase in postural vasomotor reactivity. Of the 9 patients, with diagnoses other than schizophrenia, 4 exhibited a definite increase. Of the 7 schizophrenic patients studied at least 4 months after lobotomy, 4 showed definite or marked increases (Table II). No consistent change in vasomotor reactivity developed after operation.

The schizophrenic patient showed no correlation between the presence of acral cyanosis under control conditions and increase of vasomotor reactivity; however, acral cyanosis developed, or if present, became intensified when the patients were tilted to the head-up position.

Mood or thought content changed sharply in some instances. A patient with involutional psychosis was studied 11 times; each time



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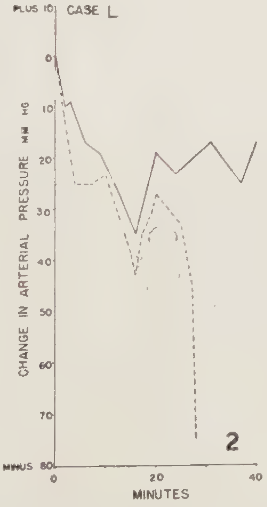

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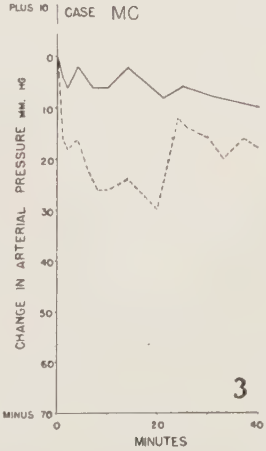

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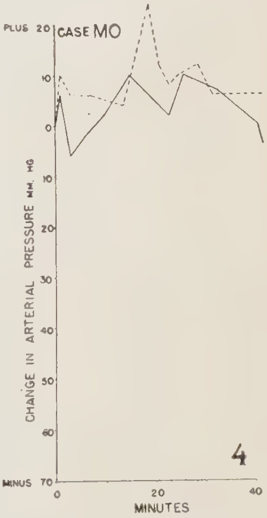

4

FIG. 1. Group I Reaction. Syncope after 3 grains of sodium nitrite.

FIG. 2. Group II Reaction. Syncope after 7 grains (dotted line) but not after 3 grains of sodium nitrite (solid line).

FIG. 3. Group III Reaction. Fall in blood pressure but no syncope after 3 grains (solid line) or 7 grains (dotted line) of sodium nitrite.

FIG. 4. Group IV Reaction. Rise in blood pressure after 3 grains (solid line) and after 7 grains (dotted line) of sodium nitrite.

distressing hallucinations of violent events developed a few minutes after she had been tilted up and when decreases in blood pressure were only slight. They persisted until she was returned to the flat position, when they disappeared, leaving the patient shaken but cognizant that the visions had been hallucinations. A patient with psychosis due to asphyxial cerebral damage became fearful when her blood pressure began to fall after she was tilted up. She spoke spontaneously for the first time of the fire during which she had almost died and which marked the onset of her illness. She described how her son had died and she also brought out other material previously suppressed. A manic-depressive patient in the manic phase became more active when her blood pressure began to fall after she was tilted up; she showed excitement and rambling speech alternating with moodiness and weeping. The patients with reactive depressions, psychoneurosis and chronic alcoholism showed no change in mood or mental content. The 15 schizophrenic patients observed showed various psychological reactions. In 3 instances syncope occurred so quickly that no evaluation of changes in thinking could be made. Two young patients with recent onset of schizophrenia became more self-absorbed and showed expressions suggestive of hallucinatory mental content when their blood pressures were falling. The remaining patients, all examples of schizophrenia of long duration, showed no definite psychological change when tilted in the head-up position although there was some decrease in blood pressure in half of them.

Discussion. The data indicate that increased reactivity of the vasomotor mechanisms involved in postural changes may exist in patients with mental and emotional disorders. This finding is not limited to schizophrenia. The mechanism of the increased vasomotor reactivity observed here is not evident. The vasomotor phenomena here observed are of interest in that they indicate the occurrence of disturbances of functions other than those concerned with thought, mood or perception in mental disease. The similarity of findings in schizophrenia and in non-schizophrenic disorders does not support the concept that the former is accompanied by a specific change in autonomic function. Moreover, the presence of increased reactivity of the postural vasomotor mechanisms in schizophrenia, negates the belief that the autonomic nervous system is less responsive than normal in this disease. The common finding of increased reactivity of vasomotor mechanisms in schizophrenic patients here and as previously discussed[1] suggests that the conclusion that such increased vasomotor reactivity is the precursor of hypertension is erroneous since the incidence of the latter is low in schizophrenia. The patients who developed activation of their psychoses when placed in the head-up position are of interest. These changes began and often became marked at a time when changes in the general circulation were too small to be noteworthy; nevertheless, at this time significant changes in cerebral circulation already must have been in progress.[2] The events in these patients suggest the wisdom of caution in ascribing the onset of, or changes in overt psychotic manifestations definitely to psychological factors whenever evidences of changes in physiological status of the brain are absent on superficial examination.

Studies made here on lobotomy, though few, indicate that it causes no distinctive change in postural vasomotor reactivity if time for complete healing is allowed. Rinkel *et al.*[3] found increases in the response to injected epinephrin in patients after lobotomy. These findings may possibly indicate overreactivity of the sympathetic nervous system, or, on the other hand, may merely indicate an altered pharmacological response to a substance given greatly in excess of physiological quantities. At any rate, the fact that Rinkel *et al.*[3] made their studies a few weeks after lobotomy, suggests that the changes they found are manifestations of an irritative phenomenon and are not due to division of the tracts cut during the operation.

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[2] Lennox, W. G., Gibbs, F. A., and Gibbs, E. L., *Arch. Neurol. and Psychiat.*, 1935, **34**, 1001.

[3] Rinkel, M., Greenblatt, M., Coon, G. P., and Solomon, H. C., *Arch. Neurol. and Psychiat.*, 1947, **58**, 570.

Summary and conclusions. In patients with mental or emotional disorders, increased activity of vasomotor mechanisms responsive to postural change may occur; this is observed irrespective of diagnosis. In some psychotic patients disturbances in mental content and mood, consisting of intensification of pre-existing psychotic manifestations, occurred at times when it may be assumed that cerebral blood flow was diminished. Observations suggest that lobotomy causes no distinctive change in postural vasomotor reactivity when time for complete healing is allowed.

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17060. Effect of Autolyzed Yeast, Yeast Nucleic Acid and Related Substances on Body-Temperatures of Rats.*

Robert M. Hill and Enid K. Rutledge.
From the Departments of Biochemistry and Pathology, University of Colorado Medical Center, Denver.

It is common practice to produce fever experimentally in rats by means of the subcutaneous injection of a solution of autolyzed yeast. During the routine use of this procedure, we found that administration of the autolyzed yeast intraperitoneally produced a fall instead of a rise in body-temperature. In the present paper we are reporting this phenomenon and some efforts to determine what constituent or constituents of the yeast may be responsible for the temperature effects. The activities of autolyzed yeast, yeast nucleic acid, and the nucleic acid derivatives. guanine, uracil, xanthine, and allantoin were investigated.

Materials. The autolyzed yeast was prepared in the usual way by making a suspension of 15 g of commercial baker's yeast in 100 cc of water. This suspension was allowed to stand for several days at 37°C with occasional shaking. The solid material was then separated in the centrifuge and the clear, dark brown, supernatant "autolysate" was used as the experimental material. The magnesium salt of yeast nucleic acid was prepared according to the method of Baumann.[1] All nucleic acid derivatives used were Eastman products and when necessary were brought to essentially neutral condition before administration.

Experimental. The experimental animals were young adult white rats about equally divided between males and females in each experiment. The animals were fasted overnight before use. High colonic temperatures were taken with mercury thermometers by means of the technic previously described.[2] As soon as the initial temperature was obtained, the substance under investigation was injected either intraperitoneally in the lower left abdominal quadrant or subcutaneously craniolaterally to the base of the tail. After injection, the animals were kept at room temperature in individual wire cages without food or water. The room temperature varied between 25.5 and 28.5°C.

With the exception of the yeast, which was given as a "15%" autolysate, all test substances were administered in 0.1% solution.

Results. Fig. 1 shows the response in temperature following the intraperitoneal injection of 2 cc of a "15%" yeast autolysate contrasted with that after the subcutaneous injection of 2 cc of the same material. The average maximum rise in body-temperature after subcutaneous administration was 1.9°C and appeared at about the 5th hour. After

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* Supported by a grant from the Office of Naval Research.

[1] Baumann, E. J., *J. Biol. Chem.*, 1918, **33**, XIV.

[2] Hill, R. M., Ware, A. G., and Schultz, F. H., *Cancer Research*, 1943, **3**, 839.

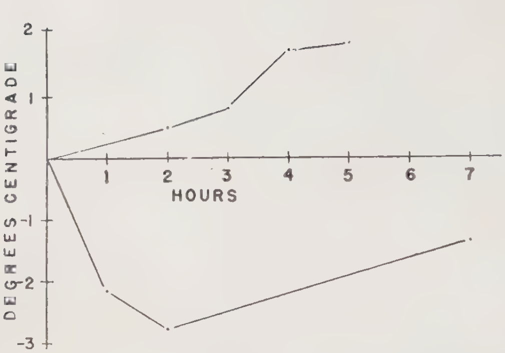

Fig. 1.
Average body-temperature change after administration of 2 cc of "15%" yeast autolysate. Upper curve, 150 rats, subcutaneous injection. Lower curve, 15 rats, intraperitoneal injection.

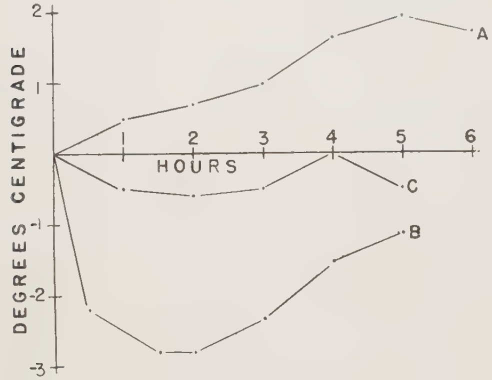

Fig. 2.
Average body-temperature change after administration of 2.5 cc of 0.1% magnesium nucleinate. A, 8 rats, subcutaneous injection. B, 8 rats, intraperitoneal injection. C, average body-temperature change after intraperitoneal injection of 2.0 cc of 0.1% guanine, 8 rats.

intraperitoneal administration, a rapid fall in body-temperature occurred, which reached an average minimum of 2.7°C below the initial value at about the 2nd hour after injection.

In Fig. 2 are shown the results of similar experiments in which 2.5 cc of a 0.1% solution of the magnesium salt of yeast nucleic acid was used. The average maximum rise in body-temperature after subcutaneous administration was 1.8°C, which occurred at about the 5th hour. The average minimum body-temperature after intraperitoneal injection was 2.9°C below the initial value and appeared after about 1.5 hours. The similarity between the results with yeast autolysate and with yeast nucleic acid is striking. Although 2.0 cc of a "15%" yeast autolysate was used on the one hand and 2.5 cc of a 0.1% nucleic acid solution was used on the other, the amount of nucleic acid injected into each animal was about the same, according to the analyses of yeast reported by Von Euler, Ahlstrom and Högberg.[3]

The effect of guanine injected intraperitoneally was slight (see the average curve in Fig. 2) and was not evident in all the animals. Guanine showed no effect when administered subcutaneously. Adenine, uracil, xanthine, and allantoin had no effect on the body-temperature by either route of administration.

During hypothermia, the rats were quiet, perhaps somewhat depressed, and showed a tendency to lie on the side. However, when they were disturbed, they reacted as quickly as untreated animals, and seemed normal in every way.

The rise in body-temperature after subcutaneous injection of autolyzed yeast or the magnesium salt of nucleic acid was slow and the peak was prolonged. The fall after intraperitoneal injection of these substances was rapid, the degree of body-temperature change was greater, and in most instances the return to the initial value occurred in a shorter time. These facts suggest that the difference in response to the two routes of administration may be due to different rates of absorption. However, very small doses given intraperitoneally do not produce hyperthermia, and large subcutaneous injections do not produce hypothermia. Another possibility is that the hypothermia following intraperitoneal injections is a manifestation of shock. Against this is the normal appearance of the peritoneal and other serosal surfaces, and the absence of visceral hyperemia, petechiae, and edema in the animals sacrificed at the time of maximum hypothermia.

Discussion. The fact that very small amounts of yeast autolysate or yeast nucleic acid given intraperitoneally did not produce hyperthermia and very large amounts given subcutaneously did not produce hypothermia would seem to rule out differences in the rate and amount of absorption as the cause of the contradictory effects on body-temperature. Our experiments imply that the property of producing hyperthermia after subcutaneous injection and hypothermia after intraperitoneal injection is inherent in the nucleic acid molecule. The fact that the large nucleic acid molecule was active though the smaller hydrolysis products of nucleic acid were not suggests that molecular or particle size may be important in causing body-temperature changes. This raises the questions of phagocytosis of the material in the peritoneal cavity and its absorption by way of the lymph channels, and the possibility that hypothermia is associated with activity of the reticulo-endothelial system. Investigations of these questions are in progress and will be reported later.

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[3] Von Euler, H., Ahlstrom, L., and Högberg, B., *Z. f. physiol. chem.*, 1942, **277**, 1.

Summary. Subcutaneous injection of 2 cc of a "15%" yeast autolysate in rats produced a significant rise in body-temperature. Intraperitoneal injection of the same material produced a significant fall in body-temperature. Both qualitatively and quantitatively similar results were produced by the injection of 2.5 cc of a 0.1% solution of the magnesium salt of yeast nucleic acid. Slight lowering of the body-temperature occurred in some animals after intraperitoneal injection of guanine. Subcutaneous injection of guanine had no effect on body-temperature. Adenine, uracil, xanthine, and allantoin had no effect on the body-temperature of rats by either route of administration.

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17061. Development of Macrocytic Erythrocytes in Leukemic Subjects Receiving Folic Acid Antagonist, 4-Aminopteroylglutamic Acid (Aminopterin).

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Temporary remissions in acute leukemia have been produced by the therapeutic administration of the folic acid antagonist 4-aminopteroylglutamic acid (aminopterin). Farber[1,2] has reported remissions in children. Dameshek[3,4] has produced equally good results in adults. The exact mechanism of the action of aminopterin is not known. It is a folic acid antagonist in that it possesses the property of inhibiting the growth of *Streptococcus faecalis R.* or *L. casei* in the presence of marginal levels of folic acid. Farber[2] noticed hypersegmentation of neutrophilic granulocytes in the peripheral blood and megaloblasts in the marrow in leukemic patients receiving folic acid antagonists. If aminopterin is a true folic acid antagonist one can theorize that morphologic changes might be produced in the erythrocytes. In a series of 25 patients (children and adults) with acute leukemia of all types treated with aminopterin, results were obtained in 7 of these individuals which support this theory.

Experimental. Daily doses of ½ to 1.0 mg of aminopterin were given intramuscularly. One cc of crude liver containing 2 U.S.P. units was given with each dose of aminopterin.

Frequent complete blood counts were done on all patients receiving therapy. Occasional bone marrow biopsies were also obtained. More complete studies were obtained when macrocytosis and anisocytosis was observed in the circulating red blood cells. These studies included red cell counts, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, morphologic observations on the erythrocytes and measurement of the mean erythrocytic diameter by the

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[1] Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., and Wolff, J. A., *New England J. Med.*, 1948, **238**, 787.

[2] Farber, S., *Blood, J. Hemat.*, 1949, **4**, 160.

[3] Dameshek, W., *Blood, J. Hemat.*, 1948, **3**, 1057.

[4] Dameshek, W., *Blood, J. Hemat.*, 1949, **4**, 168.

TABLE I.
Observations on Erythrocytes in Four Leukemic Patients Having the Most Marked Macrocytosis and Anisocytosis.

Age, yrs.	Sex	Days therapy	Total rbc.	Hgbn., g	MCH, $\gamma\gamma$	Diameter, μ	Macrocytes	Anisocytes
3	M	0	2.57	8.0	30	7.0	0	0
		30	2.91	9.0	31	7.2	++	++
		65	4.10	11.8	29	7.8	++	++
3	F	0	*5.04	14.0	28	7.2	0	0
		90	4.79	13.0	27	7.5	++	++
13	M	0	3.91	10.4	27	7.0	0	0
		15	3.22	9.3	29	7.2	+	+
		30	3.21	9.8	30	7.5	++++	++++
4	F	0	2.20	4.9	22	7.0	0	0
		32	3.78	10.5	28	8.5	++++	++++

* Transfusions.

Haden-Hausser erythrocytometer.

Observations. An obvious macrocytosis and anisocytosis developed in 7 patients. A diagnosis of acute leukemia had been made in each instance by history, examination of the patient, peripheral blood studies and bone marrow biopsies. Although it is realized that cell types in leukemic diseases of children are extremely difficult to classify it is believed that 5 cases were acute lymphatic leukemia and 2 were of the acute monocytic type. Five of the patients were under 10 years of age and 2 were in their teens.

In 4 of the 7 individuals in whom morphologic changes were observed in the red cell series a very marked macrocytosis developed. These data are presented in Table I. In no instance did a severe anemia exist. All had responded well to aminopterin and the leukemic disease had at least been controlled to the extent that no transfusions were necessary. The macrocytosis and anisocytosis in the 7 patients usually became noticeable between the fifteenth and thirtieth days of therapy. Poikilocytosis was not noticed in any of the 7 patients. The mean diameter of the red cells as measured by the erythrocytometer became greater as will be noticed in Table I. Actually an accurate evaluation of the morphologic changes in the red cells could be made by direct microscopic examination of the red cells. Photomicrographs of the red cells in 2 subjects are shown in Fig. 1. The degree of macrocytosis and anisocytosis without poikilocytosis is indeed marked.

Bone marrow biopsies were done in 2 instances when the peripheral blood showed morphologic red cell changes. Even though the peripheral blood had returned to normal and the patients were markedly improved the marrow tissue was still markedly infiltrated and replaced with leukemic cells to the extent that only rare normal bone marrow cellular elements were observed. No true megaloblasts were observed in these 2 patients. In other individuals however who have had extensive therapy with aminopterin an exceedingly rare megaloblast has been observed.

Discussion. Farber[1] in his initial report emphasized the toxic nature of aminopterin. Many of the lesions produced are similar to those produced by a folic acid deficiency in the rat and monkey. These lesions are stomatitis, ulceration of the mucous membrane of the mouth, smooth tongue, pharyngitis, and atrophic changes in the intestinal epithelium. The morphologic changes in the erythrocytes consisting of macrocytosis and anisocytosis may be possibly due to an induced folic acid deficiency. It is of interest that poikilocytosis is not present in the 7 cases reported herein. Farber[2] and Dameshek[3,4] both emphasize that it is impossible to state at this time whether all the changes produced in acute leukemia by folic acid antagonists are primarily due to a folic acid deficiency. When untoward symptoms rapidly develop that are similar to those produced experimentally, the various types of

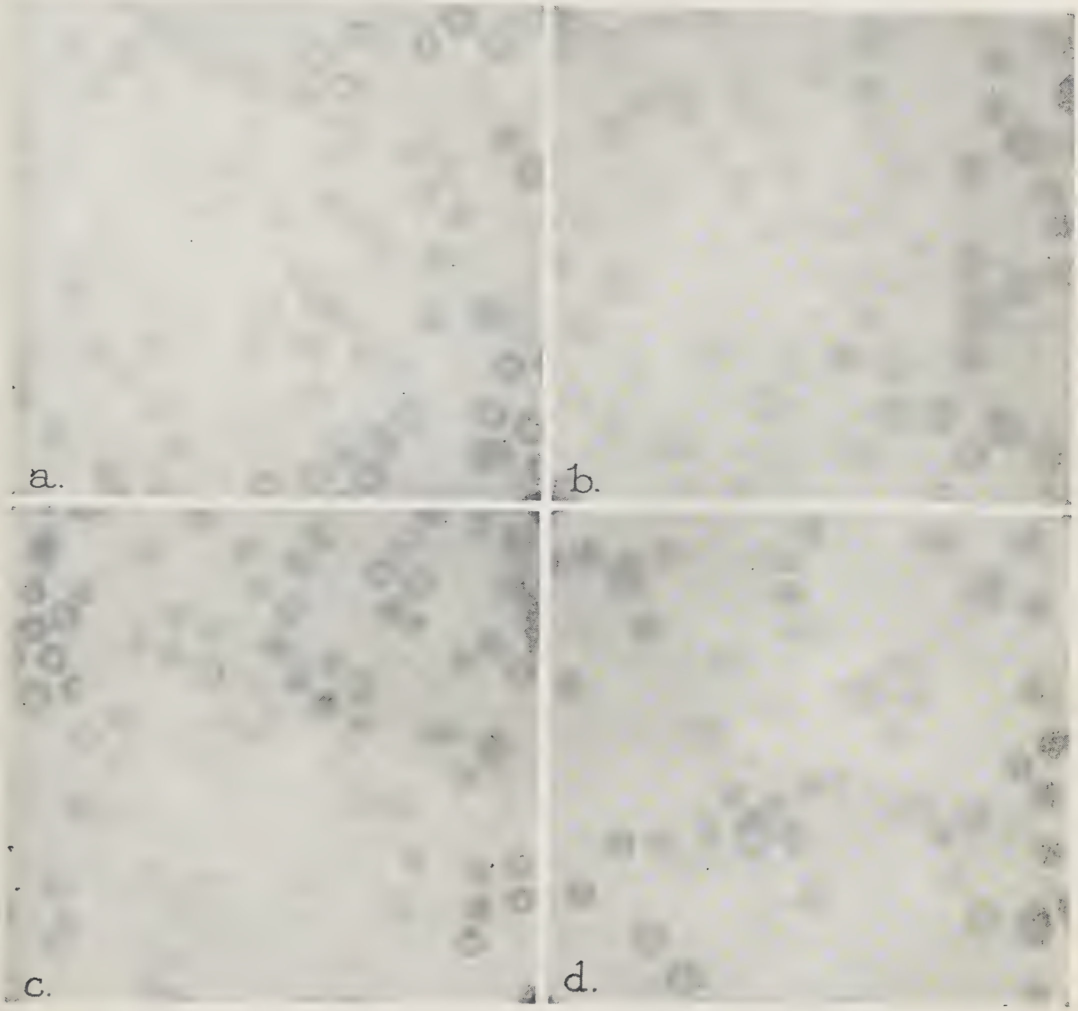

FIG. 1.
Morphologic changes in red blood cells during aminopterin therapy. A and b, patient 48-279, a, before therapy, and b, after therapy. C and d, patient 48-229, c is before therapy and d, after therapy. In each instance macrocytosis and anisocytosis are present after therapy.

therapy such as vitamin B preparations, liver extracts both crude and concentrated, and folic acid have not been more beneficial than the mere act of stopping the administration of the folic acid antagonist. Further observations on complicated biochemical systems must be made before definite conclusions can be reached.

Conclusions. In 7 of 25 human subjects with acute leukemia treated with the folic acid antagonist 4-aminopteroylglutamic acid (aminopterin) morphologic changes developed in the circulating red blood cells consisting of a macrocytosis and anisocytosis.

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17062. Effect of Heparin on Artificial Activation in the Frog Egg.*

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It is a well known fact that protoplasmic gelation precedes mitosis. The colloidal theory of cell division likens this protoplasmic gelation to blood clotting (see Heilbrunn[1] for discussion). It would be expected, therefore, on the basis of this theory that anticoagulants, such as heparin, might prevent protoplasmic gelation. Indeed, Heilbrunn and Wilson[2] have shown that this gelation in the Chaetopterus egg can be prevented by heparin and, moreover, that cell division is subsequently inhibited. Heparin also inhibits cell division in tissue cultures (Fischer[3]).

The most effective method of producing artificial parthenogenesis in the frog egg is by pricking the egg in the presence of blood or blood serum (Bataillon[4]). It was thought that the active principle in the blood might be a thrombin or thrombin-like substance which could initiate a protoplasmic gelation resulting in cell division. If this were true, anticoagulants should have some effect on parthogenesis in preventing the action of blood serum. The following experiments indicate that such is indeed the case.

Rana pipiens females were induced to ovulate by the implantation of pituitary glands, and the ovulated eggs were stripped in rows on to glass slides immediately before use. The eggs were covered with the blood mixture to be tested, and then pricked with a fine glass needle. For a discussion of the technic employed see Rugh.[5] Mixtures consisting of one part frog blood collected by heart puncture to one part heparin solution were employed in the above procedure. The heparin†

TABLE I.
% Cleavage of Eggs Pricked in 1:1 Mixtures of Blood and Heparin Made Up in Ringer's Solution.

Concentration of Heparin in g %						
0.0	0.2	0.4	0.8	1.6	2.4	3.2
68	60	—	—	—	—	—
41	—	25	19	—	—	—
63	—	—	54	42	—	—
46	38	40	43	24	—	—
62	56	55	56	46	—	—
70	—	—	51	50	—	56
67	—	—	—	66	—	63
72	—	—	69	72	—	65
61	—	—	44	48	—	44
64	—	—	40	43	46	34
49	42	48	—	—	—	—
61	—	45	38	45	48	32
76	63	74	—	—	69	62
77	72	67	—	—	65	55
77	—	—	—	—	66	—
67	61	65	—	—	63	60
59	39	23	—	—	31	—
68	68	54	—	—	60	—
51	29	—	—	—	26	—
43	—	—	—	—	32	—
64	—	—	36	45	—	38

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* Aided by a grant from the U. S. Public Health Service, administered by L. V. Heilbrunn.

[1] Heilbrunn, L. V., *An Outline of General Physiology*, 2nd ed., Philadelphia, 1943.

[2] Heilbrunn, L. V., and Wilson, W. L., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 179.

[3] Fischer, A., *Arch. f. path. Anat. u. Physiol.*, 1930, **279**, 94.

[4] Bataillon, E., *Compt. rend. Acad. Sci. Paris*, 1911, **152**, 920.

[5] Rugh, R., *Experimental Embryology*, New York, 1941.

was made up in frog Ringer's solution in concentrations of 0.2, 0.4, 0.8, 1.6, 2.4, and 3.2 grams percent. Control eggs were covered with a mixture of one part frog blood to one part Ringer's. A third set of eggs were pricked in the absence of any solution. In this way it was possible to compare the effectiveness in producing artificial parthenogenesis of heparinated blood with the effectiveness of plain blood of the same concentration. After treatment the eggs were immediately transferred to pond water which had been boiled and filtered, and were kept at room temperature. Four to 5 hours later, counts were made of the number of divided cells and the total number of cells. No attempt was made to distinguish between normal and abnormal divisions.

The results are summarized in Table I, which shows the percent cleavage obtained when the eggs were pricked in the presence of blood and Ringer's fluid (0% heparin in column 1), and in the presence of blood and heparin of various concentrations (columns 2-7). In every case the percent of dividing eggs was lower when the heparinated blood was used than when the blood-Ringer's solution was used. In only one case, however, was the percent as low as in eggs treated by pricking alone. The values obtained for the experimental eggs were calculated as percent of the blood-Ringer's controls, averaged for each concentration, and they were then plotted in Fig. 1. The 100% line represents the cleavages in the blood-Ringer's fluid. From this figure it can be seen that there is a definite inhibition by heparin of activation by pricking in the presence of blood. This lends further support to the colloidal theory of cell division.

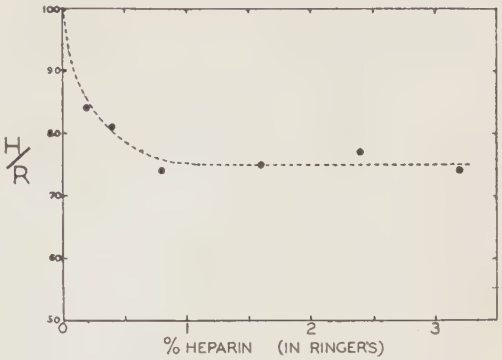

Fig. 1.

Inhibition by Heparin of Artificial Activation.

$$H/R = \frac{\%\ \text{Cleavage in Blood-Heparin Solution}}{\%\ \text{Cleavage in Blood-Ringer's Solution}} \times 100$$

Summary. Eggs of *Rana pipiens* were pricked in the presence of heparinated frog blood. The percent of dividing eggs was lower in every case than when the eggs were pricked in the presence of blood and Ringer's fluid

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† Hynson, Westcott and Dunning, lots 198 and 202.

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17063. Failure of Rutin to Inhibit Hyaluronidase in the Albino Rat.

Samuel K. Elster. (Introduced by Joel Warren.)

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Rutin, a naturally occurring flavanol glucoside, has been found to influence favorably various states characterized by an increased capillary fragility.[1] It has been demonstrated that hyaluronidase increased the speed of passage of fluids and the blue dye T-1824 across the capillary membrane in the albino rat.[2] Chambers and Zweifach[3] have considered that hyaluronidase accentuated capillary fragility rather than produced direct changes in capillary permeability. *In vitro,* rutin in high concentrations effectively inhibited hyaluronidase, but was inactive in lower concentrations.[4] Levitan[5] tested this reaction *in vivo* in the albino rat, and concluded that rutin markedly inhibited the spreading activity of intradermally injected inked hyaluronidase.

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[1] Griffith, J. Q., Jr., Couch, J. F., and Lindauer, M. A., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 228.

[2] Elster, S. K., Freeman, M. E., Dorfman, A., *Am. J. Physiol.*, 1949, in press.

[3] Chambers, R., and Zweifach, B. W., *Physiol. Rev.*, 1947, **27**, 436.

A technic has been described[6] in which the activity of hyaluronidase was tested in the albino rat. Following the intravenous administration of hyaluronidase, changes in blood volume occurred. Edema of the extremities appeared and marked elevation of the blood hematocrit was noted. A substance may be tested for its ability to inhibit this reaction, if it does not of itself alter the fluid balance of the body. Thus, a substance may be considered a hyaluronidase antagonist if, after its administration, the hematocrit does not rise following the injection of the standard amount of enzyme.

This technic has been applied to the study of rutin in the body and its possible role in hyaluronidase inhibition.

Experimental. 136 male albino rats of the Sherman strain were fed Purina laboratory chow and water *ad lib.* The hyaluronidase was prepared according to the method of Freeman *et al.*[7] The enzyme was assayed[8] and measured 3000 turbidity reducing units (T.R.U.) per mg of nitrogen.

1) *The effect of the route and mode of administration of rutin on the hematocrit.* 64 animals, weighing 200-350 g were divided into 5 groups:

Group A—20 normal rats.

Group B—15 rats, 200 mg rutin suspended in 1 ml normal saline fed orally (via stomach tube) for 4 successive days.

Group C—9 rats, 200 mg rutin suspended in 1 ml normal saline injected intraperitoneally.

Group D—10 rats, 1 ml propylene glycol injected intraperitoneally.

Group E—10 rats, 200 mg rutin dissolved in 1 ml propylene glycol injected intraperitoneally.

All animals were bled from the aorta and sacrificed. The hematocrit was determined by centrifugation at 3000 rpm for 30 minutes.[9] Blood was drawn from Groups C, D and E 3½ hours following the injections and from Group B 1½ hours following the last gastric administration.

Marked changes were noted in the animals in Groups C, D and E. They were lethargic and their abdomens were protuberant. When their abdominal cavities were opened, several ml of free fluid were encountered. In Groups C and E, this fluid was light yellow; rutin was precipitated on the peritoneal surfaces of the organs. The abdominal fluid encountered in the animals of Group D was slightly cloudy and colorless. The intestines and peritoneum were inflamed and edematous. The most severe changes were noted in the animals of Group E, in which as much as 8 ml of fluid were removed from the abdominal cavity. The animals of Groups A and B appeared normal.

The changes in plasma volume are reflected in the hematocrit determinations (Fig. 1).

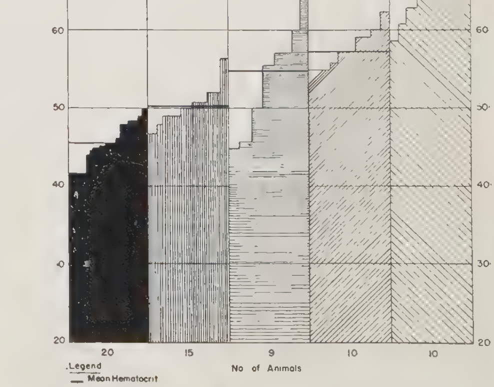

FIG. 1.

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[4] Beiler, J. M., and Martin, G. J., *J.B.C.*, 1947, **171**, 507.

[5] Levitan, B. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 566.

[6] Elster, S. K., Freeman, M. E., Anderson, P., *J. Lab. and Clin. Med.*, 1949, in press.

[7] Freeman, M. E., Anderson, P., Oberg, M., and Dorfman, A., 1949, in press.

[8] Dorfman, A., and Ott, M. L., *J.B.C.*, 1948, **172**, 367.

[9] Wintrobe, M. M., Clinical Hematology, Lea & Febiger, 1947, Philadelphia.

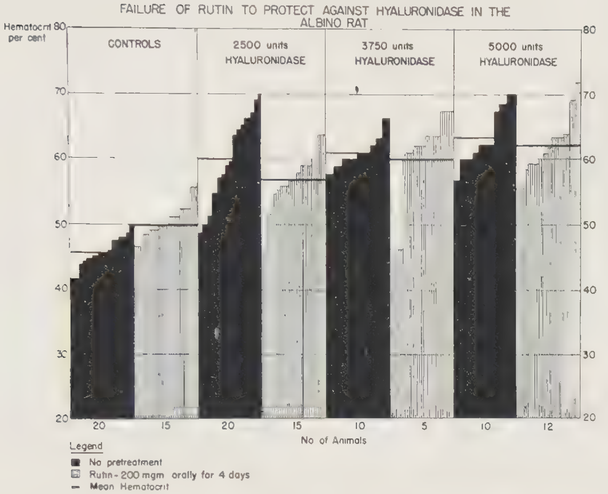

Fig. 2.

The mean hematocrit of Group A, 46.0%, was representative of normal values obtained for a similarly constituted normal population of 150 male rats. All other groups showed evidence of hemoconcentration. The mean hematocrit value for Group C was 54.4%, Group D 57.1% and Group E 68.5%. A few animals of Group C had a normal hematocrit, but none of Group D or E was normal. Group E had the greatest clinical effects and the greatest hemoconcentration. Every animal of this group had severe diminution of plasma volume. The hematocrits of the rats in Group B were slightly elevated; the mean value was 50.3%. Most animals of this group had little or no change of hematocrit and only one had a moderate increase (56%). Therefore, the oral route was selected as the best available means of rutin administration for the second part of the experiment, since it caused the least change in the hematocrit.

2) *The role of rutin in hyaluronidase antagonism in vivo.* 72 animals weighing 160-230 g were used. Thirty-two animals were fed 200 mg rutin in 1 ml saline by stomach tube for 4 successive days. On the last day 1½ hours following the gastric administration, 15 rats were given 2500 T.R.U., 5 rats were given 3750 T.R.U. and 12 animals were given 5000 T.R.U. hyaluronidase by the intravenous route. At the end of 30 minutes, they were bled from the aorta and the hematocrit of the blood determined. Forty animals were not pretreated with rutin; 20 rats were given 2500 T.R.U., 10 rats were administered 3750 T.R.U. and 10 were given 5000 T.R.U. hyaluronidase intravenously. These were bled and sacrificed 30 minutes after the injection and the blood hematocrit determined.

The animals that received hyaluronidase alone, developed edema of the extremities and face within 5-15 minutes; this was maximal in 30 minutes. In a previous communication[2] it has been reported that the edema began to recede in 2 hours and had disappeared completely in 24 hours. The hematocrits of the blood were markedly elevated (Fig. 2); the mean values were 60.0 to 62.9%. The group that was pretreated with rutin and then received hyaluronidase intravenously was indistinguishable from the previous group. Edema of equal intensity and duration appeared in the same areas. The hematocrits of these animals were elevated and the mean values were not significantly different from that group receiving hyaluronidase alone. Although the range of values was different, the mean hematocrit (57.1%) of the animals receiving 2500 units hyaluronidase and rutin was not statistically different from the mean (60.0%) of the group receiving only hyaluronidase. In the groups receiving the larger amounts of hyaluronidase, only one animal failed to respond with a rise of hematocrit.

Discussion. In the evaluation of the inhibitory action of rutin on hyaluronidase *in vivo,* the method of rutin administration must be considered. It has been demonstrated that 200 mg rutin suspended in saline, when given intraperitoneally to a rat, acted as an irritant and caused the formation of hydroperitoneum. Propylene glycol had a similar action. Rutin dissolved in propylene glycol was the most active and caused so much peritoneal fluid loss that marked hemoconcentration occurred. In the experiments of Levitan, 200 mg rutin dissolved in 1 ml propylene glycol were given intraperitoneally to rats weighing 250-400 g. Three and one-half hours later, testicular hyaluronidase and india ink were injected into the skin and the rats were sacrificed 22 hours after that. There was marked inhibition of the spread of the hyaluronidase-ink mixture, as well as a decrease in the spread of the control ink and saline. Propylene glycol alone, when given intraperitoneally, caused a smaller decrease in the spread of the hyaluronidase. Since both of these agents have been demonstrated to cause extreme hemoconcentration when given intraperitoneally, the validity of conclusions as to the efficacy of these drugs on hyaluronidase inhibition is open to extreme doubt. Duran-Reynals[10] has emphasized that "in the living animal a permeating effect on blood capillaries takes place which may contribute to the final phase of the spreading." In an animal in a condition of marked diminution of blood volume, the available fluid for the spreading reaction is greatly diminished and the conditions for spread are not optimal.

Rutin has been found to inhibit hyaluronidase *in vitro,* and this inhibition has been potentiated by Vitamin C. These studies have demonstrated that rutin, when administered orally, failed to modify the action of hyaluronidase on edema formation and elevation of hematocrit in the albino rat. Rutin in the body is presumed to decrease capillary fragility. However, the investigations performed at this laboratory have failed to produce evidence that hyaluronidase increased capillary fragility, as the edema is not accompanied by red blood cell extravasation.

Summary. 1. Hyaluronidase increased the diffusion of fluid across the capillary membrane and cause elevation of the blood hematocrit.

2. Rutin, in the amounts used in these experiments, failed to inhibit this reaction in the albino rat.

3. Rutin in propylene glycol, given intraperitoneally to the albino rat, caused the formation of hydroperitoneum and marked hemoconcentration. Propylene glycol given by the same route had a similar, although lesser, effect. When administered orally, rutin caused little elevation of the hematocrit.

The assistance of Miss Elaine Lowry is gratefully acknowledged. The author is indebted to Major Monroe E. Freeman, MSC, for the hyaluronidase preparations.

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[10] Duran-Reynals, F., *Bact. Rev.*, 1942, **6**, 197.

17064. Role of Potassium in Dialysing Fluid in Treatment with the Artificial Kidney.

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Spontaneous elevations of serum potassium levels with electrocardiographic changes similar to those found in dogs with experimental anuria[1] have been demonstrated in man during the terminal stages of uremia,[2-6] indicating that hyperpotassemia may be an important

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[*] C. Rutherfurd Caverhill Fellow in Medicine, McGill University.

[†] Archibald Fellow in the Department of Experimental Surgery, McGill University.

[1] Hoff, H. E., Smith, P. K., and Winkler, A. W., *J. Clin. Invest.*, 1941, **20**, 607.

TABLE I.

Concentration of Potassium in Patient's Serum and in the Dialysing Fluid Before and After Dialysis.

	Serum K			K of dialysing fluid		
Case No.	Before dialysis, mg %	After dialysis, mg %	Length of treatment, hrs	Before treatment, mg %	After treatment, mg %	Amt of K removed by dialysis, g
I	26.3	23.7	6	20.1	20.9	.80
II	28.3	24.6	6	5.0	12.6	7.6
III	20.4	11.9	6*	7.76	7.56†	—

* After 4 hours 35 g of KCl was added to the dialysing fluid because of the changes in the electrocardiogram, indicating hypopotassemia. The potassium content of the dialysing fluid was hereby increased to 24.5 mg %. Treatment was then continued for another 2 hours.

† Potassium content of the dialysing fluid after the first 4 hours of treatment before the 35 g of KCl was added.

factor in the mechanism of death in uremia, especially when associated with anuria or oliguria.

This study was undertaken to estimate the use of an artificial kidney in the elimination of potassium from the body in cases of uremia in which the serum potassium levels are elevated.

Methods. Serum potassium was determined and electrocardiograms were taken in 3 patients with a severe degree of uremia, prior to, during, and after treatment with the artificial kidney. The artificial kidney and the constitution of the dialysing fluid were similar to those used by Kolff,[7] but in cases II and III no KCl was added to the dialysing fluid. The potassium in the dialysing fluid was estimated before and after treatment. Its level of 5 and 7.76 mg% in the dialysing fluid before treatment in cases II and III respectively (Table I) was probably due to potassium contaminants in the other salts and the tap water in the dialysing fluid. The other biochemical and clinical findings in these cases will be reported in detail elsewhere.[8,9]

Results. From the experimental data in Table I, it is apparent that when the potassium content of the dialysing fluid is below 20 mg% (see case II) a significant amount of potassium can be removed by artificial dialysis from the body of uremic patients with hyperpotassemia, without danger of hypopotassemia. When the potassium of the dialysing fluid is maintained at 20.1 mg% (case I) the serum potassium is lowered only slightly and an insignificant amount of potassium is actually removed from the body. In uremic patients with normal serum potassium levels, hypopotassemia can occur when the potassium of the dialysing fluid is markedly lowered (case III, Table I).

In case III, changes in the electrocardiogram indicative of some degree of hypopotassemia[10] were observed after 4 hours of dialysis and potassium was then added to the dialysing fluid and the dialysis continued for another 2 hours. Although the serum of the blood withdrawn at this time was found to contain 11.9 mg% of potassium, the patient did not show clinical symptoms associated with hypopotassemia. During the dialysis the patient voided 1,200 cc of urine. The serum potassium level 5 hours after the dialysis was 14.6 mg%.

Discussion. Kolff[7] originally omitted potassium from the dialysing fluid, but owing

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[2] Marchand, J. F., and Finch, C. A., *Arch. Int. Med.*, 1944, **73**, 384.

[3] Keith, N. M., Burchell, H. B., and Baggenstoss, A. H., *Am. Heart J.*, 1944, **27**, 817.

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[5] Bywaters, E. G. L., *J.A.M.A.*, 1944, **124**, 1103.

[6] Strauss, M. B., *New England J. Med.*, 1948, **239**, 693.

[7] Kolff, W. J., "New Ways of Treating Uremia," J. & A. Churchill, Ltd., London, 1947.

[8] Miller, G. G., and Ripstein, C. B., to be published.

[9] Wener, J., de Leeuw, N. K. M., and MacLean, J. T., to be published.

[10] Martin, H. E., and Wertman, M., *Am. Heart J.*, 1947, **34**, 646.

to the possible dangers of producing hypopotassemia, he and later Bywaters and Joekes,[11] both recommended that physiological amounts of potassium be included in the dialysing fluid.

From Kolff's[7] data it was apparent that hypopotassemia occurred only in a patient who had a normal serum potassium concentration to start with, very similar to that in case III, Table I.

The variable results obtained in these cases are probably due to the differences in the direction of the potassium shift. In case II, the increase of 7.6 g of potassium in the dialysing fluid during the dialysis indicated that potassium was removed from the intracellular as well as from the extracellular fluids. In cases I and III, since there was no significant increase of potassium in the dialysing fluid, the potassium shift probably occurred mainly from the extracellular to the intracellular fluids. The lowering of the serum potassium in these instances might be explained by other factors: (a) the dilution of the patient's blood with the blood added to the artificial kidney prior to its use, and by the further addition of varying amounts of blood and intravenous fluids during the treatment: (b) the fact that the glucose present in very high amount[7,11] in the blood leaving the artificial kidney to be returned to the body will, on entering the systemic circulation, be stored as glycogen, with the resultant shift of potassium from the extracellular to the intracellular spaces[10] was the main reason that Bywaters and Joekes[11] recommended that physiological amounts of potassium to be added to the dialysing fluid; and (c) potassium elimination from the blood stream by urinary excretion. This last factor may have operated in Case III where the patient voided 1,200 cc urine during the period of dialysis.

Summary. From the observations reported it would appear that the artificial kidney can serve as a safe and efficient method for removing excess potassium from the body in uremia patients with hyperpotassemia. However, further study is required to determine the amount of potassium that should be added to the dialysing fluid in each individual case. This will probably vary with the degree of hyperpotassemia present before treatment is begun.

We wish to express our thanks to Doctors G. G. Miller and J. T. MacLean for permission to study their patients. We are grateful to Dr. Martin Hoffmon for many helpful suggestions and criticisms.

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[11] Bywaters, E. G. L., and Joekes, A. M., *Proc. Royal Soc. Med.*, 1948, **41**, 420.

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17065. Nu 445 in the Treatment of Urinary Infections Due to Gram Negative Bacilli.*

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Introduction. Nu 445† (3,4-dimethyl-5-sulfanilamido-isoxasole) is one of the newer sulfonamides said to be highly effective for urinary tract infections due to *E. coli* and *B. proteus*.[1-6] The reported results on the therapeutic activity of this drug in man are meager

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* Aided by a grant from the Patrons of Research, Beth Israel Hospital.

† Supplied through the courtesy of Hoffmann-La Roche, Inc.

[1] Schnitzer, R. J., Foster, H. K., Ercoli, N., Soo-Hoo, A., Mangieri, C. N., Roe, M. D., *J. Pharm. and Exp. Therap.*, 1946, **88**, 47.

[2] Sarnoff, S. J., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 23.

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[4] Sarnoff, S. J., Freedman, M. A., Hyman, A. A., *J. Urol.*, 1946, **55**, 417.

TABLE I.
Comparative *in vitro* Sensitivity of Several Strains of Various Gram Negative Micro-organisms to 4 Sulfonamides.

Strain	Nu 445‡ titers in mg %		ST‡ titers in mg %		SD‡ titers in mg %		SMT‡ titers in mg %	
	c*	st†	c	st	c	st	c	st
E. coli 1	750	500	500	100	250	50	25	25
,, 2	1000	500	500	100	250	100	250	100
,, 3	750	500	1000	1000	1000	250	50	25
,, 4	100	25	500	250	500	250	750	100
,, 5	250	100	500	100	500	25	500	250
A. aerogenes 1	750	500	500	100	>1000	>1000	750	500
,, 2	>1000	>1000	1000	750	>1000	1000	250	100
,, 3	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
,, 4	1000	500	500	100	>1000	1000	750	500
B. proteus 1	>1000	500	750	50	250	25	100	25
,, 2	>1000	>1000	1000	500	>1000	>1000	500	100
,, 3	>1000	>1000	>1000	>1000	>1000	>1000	1000	750
,, 4	250	100	1000	500	>1000	>1000	750	250
B. pyocyaneus 1	>1000	>1000	>1000	1000	>1000	>1000	750	500
,, 2	500	100	>1000	750	>1000	>1000	1000	500
,, 3	>1000	>1000	500	100	>1000	>1000	750	500
,, 4	>1000	1000	>1000	>1000	>1000	>1000	500	100
E. typhi 1	1000	250	500	250	1000	500	250	50
,, 2	750	250	500	100	1000	500	500	100
S. paratyphi 1	1000	250	500	250	>1000	>1000	250	25
,, 2	1000	250	750	500	>1000	>1000	250	100
S. schottmuelleri 1	>1000	1000	>1000	750	>1000	>1000	500	100
,, 2	>1000	1000	>1000	750	>1000	750	500	100

* c = bactericidal titer.
† st = bacteriostatic titer.
‡ NU 445—3,4-dimethyl-5-sulfanilamido-isoxasole.
ST —sulfathiazole.
SD —sulfadiazine.
SMT —sulfamethazine.

and conflicting. *In vitro* and *in vivo* studies on animals showed that sulfadiazine was slightly superior to Nu 445 against *E. coli,* moderately superior against *S. schottmuelleri* and greatly superior against *Klebsiella A.*[1] Because of these inconsistencies, a further clinical and *in vitro* study of the drug is herewith reported.

In vitro studies: The *in vitro* action of the drug on various gram negative bacilli was determined and compared with that of sulfadiazine (SD), sulfamethazine (SMT), and sulfathiazole (ST).

Serial dilutions of a 1% solution of the drug in nutrient broth (pH 8.2) were made, and to 0.9 cc of each dilution was added 0.1 cc (500 to 3000 microorganisms) of a bacterial suspension from a 24-hour growth. The highest drug dilution at which there was no macroscopically visible growth after 24 hours at 37° was taken to be the bactericidal titer (c). The highest drug dilution in which there was definitely less turbidity as compared to the control tubes was taken to be the bacteriostatic titer (st).

Most of the bacterial strains tested were freshly isolated from infected urines. *E. typhi* and the *Salmonellae* had been cultured from stools or blood and had undergone a number of transfers.

Table I shows that the 4 sulfonamides test-

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[5] Rodgers, R. S., Colby, F. H., *J. Urol.*, 1948, **59**, 659.

[6] Haines, W. H., Micelli, S., *Penn. Med. J.*, 1947, **50**, 1328.

TABLE II.
Results of Treatment with Nu 445 in Uncomplicated* and Complicated† Urinary Infections.

Type of disease	Type of infection	No. of cases	Cases cured, No.	Cases not cured, No.
Uncomplicated	Monovalent Polyvalent	7 2	6	3
Complicated	Monovalent Polyvalent	1 10	1	10
Total		20	7	13

* Uncomplicated means acute cystitis, acute pyelitis, or acute pyelonephritis without any additional pathology.

† Complicated means additional pathology such as severe chronic infection, calculi, hydronephrosis, obstruction to urinary flow, or constant drainage.

ed exert differing effects on various strains of the same species of bacteria. For example, of the 5 *E. coli* strains tested, 2 were most sensitive to SMT, 2 to Nu 445, and one equally to SD and SMT. Of 4 strains of *Ps. aeruginosa, (B. pyocyaneus)*, 3 were best inhibited by SMT, one by Nu 445. The effect of SMT on the typhoid-paratyphoid group was definitely superior to that of the other 3 drugs. From the foregoing data, Nu 445 appeared to be worthy of clinical trial in infections due to *E. coli, B. proteus*, or *Ps. aeruginosa.*

Clinical Observations. Twenty unselected patients with a variety of urinary tract infections due to gram negative bacilli were given Nu 445 orally. In spite of large daily doses, the blood levels remained rather low indicating rapid acetylation and excretion of the drug.[5]

The results of treatment are indicated by 2 types of response. A. Clinical and bacteriological cure. This result was obtained in 7 patients (35%). The patients in this group were followed for at least 2 weeks, and at least 2 sterile cultures were obtained after termination of treatment. B. Clinical and bacteriological failure. Of the 13 patients (65%) in this group, 3 showed temporary improvement, but had a recurrence shortly after the drug was omitted.

Discussion. The results of treatment with Nu 445 are inferior to those obtained with SMT and other sulfonamides.[7-12] Six of 7 patients with pure *E. coli* infection and one patient with pure *B. proteus* infection were cured. Four of these had received SD without benefit. Not one of 12 patients with mixed infection was cured. Three, however, for various reasons, did not receive a full course of the drug. Ten of the 12 had complicating pathology such as obstruction, calculi, hydronephrosis or constant drainage, etc. (Table II).

Although alkali therapy was not administered, the urine pH ranged from 4.5-7.5. No renal damage or crystalluria was observed in any patient. In 2 patients a dose of 12 g daily for several days and in one patient a dose of 19 g inadvertently given in one day, did not produce any toxic symptoms. Mild nausea and vomiting occurred once after 6 g had been given daily for 4 days, and disappeared within 24 hours after termination of therapy.

Nu 445 is a safe and effective drug in uncomplicated monovalent infections of the urinary tract due to *B. proteus* or *E. coli*, but is of little or no value in polyvalent infections, and in those associated with complicating organic disease in which SD[8] and SMT[7] have occasionally achieved cure. However, Nu 445, like other sulfonamides and antibiotics, may produce a cure when other drugs do not.

Summary. 1. An *in vitro* study of the action of Nu 445 shows that this drug occasionally exerts a greater bacteriostatic and bactericidal effect on certain strains of gram negative bacilli than do other sulfonamides.

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[10] Helmholz, H. F., *Proc. Staff Meetings, Mayo Clinic*, 1942, **17**, 529.

[11] Culp, O. S., *J. Urol.*, 1940, **44**, 116.

[12] Alyea, E. P., *J. Urol.*, 1942, **47**, 219.

2. Clinical trial in 20 patients with urinary infections indicated excellent effectiveness of the drug in uncomplicated, monovalent infections due to *E. coli* or *B. proteus*.

3. Results of treatment in polyvalent infections and in cases with complicating pathology were poor.

4. The marked lack of toxicity of Nu 445 recommends its use in appropriate cases, in which it will occasionally be found to be more effective than other sulfonamides.

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17066. Metabolic Fate of 4,4'-Diaminodiphenylsulfone (DDS) in the Rabbit and its Isolation from Urine.

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4,4'-Diaminodiphenylsulfone (DDS) and many of its derivatives have been used with a measure of success in the treatment of experimental tuberculosis.[1,2] More recently the potentiating action of some sulfone derivatives on streptomycin has also been shown.[3] Of the various types of sulfone derivatives indirect evidence has indicated that certain disubstituted derivatives like promin, diasone and possibly sulphetrone are metabolized to the toxic parent substance DDS while the monosubstituted alkyl and hydroxyalkyl derivatives do not appear to undergo such transformation in the body to an appreciable extent.[4] Indirect evidence has also indicated that DDS itself does not change to any significant degree in the body, at least in so far as acetylation is concerned.[1,5] Obviously, direct evidence on these points can only be had by the actual isolation of DDS from the urine. The present report describes a satisfactory method for the isolation and recovery of DDS added to rabbit urine and the isolation and the identification as DDS of about 70% of the total diazotizable material in the urine of rabbits given DDS orally.

Procedure.. The isolation of DDS from rabbit urine is based on the solubility of its hydrochloride in water and the relative insolubility of the base in water, but its ready solubility in ethyl acetate. Ethyl acetate appears to be the most satisfactory of several water-immiscible solvents tried.

The urine is made strongly alkaline to litmus with NaOH and chilled on ice for a while to aid precipitation of the base. It is then shaken out thoroughly 2 to 3 times in a separatory funnel with 30 to 50 cc of ethyl acetate, allowing as complete separation as possible each time. The partly emulsified combined ethyl acetate extract is cleared by the gradual addition of anhydrous sodium sulfate with stirring. The clear ethyl acetate solution is decanted and the Na_2SO_4 residue is washed 2 to 3 times with small portions of ethyl acetate.

The combined ethyl acetate extracts are washed once with about 5 cc H_2O and then thoroughly extracted with 5 cc portions of N HCl. This should be repeated 5 to 7 times. The combined acid extract is chilled on ice

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* Laboratory of Tropical Diseases, Microbiological Institute.

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[4] Smith, M. I., Jackson, E. L., Junge, J. M., and Bhattacharya, B. K., *Am. Rev. Tuberc.*, in press.

[5] Smith, M. I., and McClosky, W. T., *Am. Rev. Tuberc.*, 1945, **52**, 304.

TABLE I.

Fractionation of Rabbit Urine for the Isolation of 4,4'-Diaminodiphenylsulfone (DDS). Figures in first 4 fractions give DDS equivalent in mg as obtained by the Bratton and Marshall Diazotization Technic.

		Urine of rabbits given 0.5 g/kg DDS orally		
Fraction	30 cc normal urine with 63 mg DDS added	30 cc urine containing 100 mg DDS equivalent	30 cc urine containing 100 mg DDS equivalent	60 cc urine containing 200 mg DDS equivalent
1. Residual urine after extraction with ethyl acetate	0.25	7.2	8.8	10.3
2. Ethyl acetate ext. after extraction with N HCl evaporation to dryness, residue taken up in dil HCl and filtered	0.25	4.8	5.8	7.1
3. Mother liquor and washings of crystals obtained by alkalinization of the N HCl extract	9.0	20.3	15.6	20.9
4. Acetone insoluble part of isolated crystals	trace	trace	trace	—
5. Acetone soluble part of isolated crystals				
(a) Weight mg	47	63	68	141
(b) % of total	74.6	63	68	70.5
(c) Melting point °C	175-6	170-2	170-2	171-2
(d) M.P. when mixed with pure DDS °C	175-6	173-4	173-4	174-5

and alkalinized by addition of about 20% NaOH solution. After being chilled on ice the crystals are collected by centrifugation, washed by centrifugation with a minimum volume of ice cold water until neutral to litmus and dried at 60-80°C. The dry residue in the centrifuge tube is dissolved for the most part in a few cc of acetone, centrifuged, the clear solution decanted into a suitable weighing bottle and the operation repeated 2 to 3 times, to separate the acetone-soluble part from the insoluble solid which is discarded. The acetone is allowed to evaporate and the residue dried *in vacuo* to constant weight. The material so obtained from urine, when pure DDS is added to it, had a sharp melting point of 175-176°C. Material so obtained from urine of rabbits given DDS *per os* assayed 100% by a modification of the Bratton and Marshal diazotization technic and usually had a melting point of 170-172°C; when mixed with pure DDS it melted at 173-175°C. Recrystallization of this material by dissolving it in a minimum volume of acetone and slowly adding 5 to 6 volumes of water, centrifugation and drying yielded crystals which had a melting point of 172-174°C. The crystals were decolorized in hot methanol solution with Norit and recovered by evaporation of the solvent. A final recrystallization from a little methanol yielded DDS melting at 175-176°C alone or mixed with authentic crystals of DDS.

Table I shows the results of fractionation of a sample rabbit urine to which 63 mg of pure DDS had been added as the hydrochloride and similar fractionation of pooled catheterized specimens of urine obtained from 3 rabbits 3 to 6 hours after the oral administration of 0.5 g/kg of DDS in aqueous suspension with gum acacia. In the latter instance the urine was assayed by a modified Bratton and Marshal technic[6] against DDS as a standard and a volume of urine corresponding to the amount of DDS equivalent indicated in the table was used for fractionation.

The data in Table I indicate that at least 63-70% of the total diazotizable material in the urine is unaltered DDS. Parallel assays of the several fractions by diazotization after acid hydrolysis at 100°C and direct diazotization at room temperature gave identical values except in fraction one in which the former values were from 10-30% higher, suggesting that a small amount, not over 5% of the total, had been altered to some metabolite, possibly conjugated DDS.

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The technic of partition chromatography on filter paper, as originated by Consden, Gordon and Martin[7] has been adapted for the resolution of DDS and several synthetic derivatives from mixtures. Normal butanol saturated with 3% ammonia, benzene saturated with water, and equal parts of benzene and cyclohexane saturated with water were used with ascending columns of narrow (⅛ in. wide) strips of Whatman's No. 1 filter paper. The papers were hung for 3 to 24 hours in a liter graduate cylinder containing 100 cc of one of the above solvents. Colored bands of DDS or monosubstituted derivatives were produced by pipetting the Bratton and Marshal reagents (dissolved in organic solvents to minimize dispersal of the pigments produced) on the dried strips. The p-dimethylaminobenzaldehyde reagent (1% in 3% HCl) of Kühnau,[8] giving a yellow to orange precipitate, has also been found useful. It has been found that rabbit urine containing DDS or certain derivatives, may be chromatographed without prior treatment. Aliquots of 0.01 cc, containing 0.1 to 1.0 γ of each sulfone, were used.

Applying such methods to the several fractions in Table I evidence has been obtained to indicate that, in addition to the original urine, fraction 1 and possibly also fraction 2 contained another compound besides DDS. The original urine and fraction 1 gave 2 distinct bands when chromatographed from the benzene-water solvent. One of these bands, the upper, had the same R_F value as pure DDS. The other, the lower band ($R_F < 0.1$), was unlike those produced by the monoacetyl derivative of DDS or 3-hydroxy-4,4′-diaminodiphenylsulfone or the sulfide and sulfoxide analogues of DDS.

These results demonstrate that the bulk of the diazotizable material excreted in the urine following the oral administration of DDS to rabbits is unchanged DDS. The procedure described also provides a ready means for determining whether a given derivative of DDS is metabolized to the parent substance or not.

Summary. A method is described for the isolation and identification of 4,4′-diaminodiphenylsulfone (DDS) in the urine of rabbits. The method is based on the solubility of the hydrochloride of DDS in water and the relative insolubility of the base in water but its ready solubility in ethyl acetate.

Up to 70% of the total diazotizable material excreted in the urine following oral administration of DDS to rabbits has been isolated as unchanged DDS. Not more than about 5% of the diazotizable material may be in an altered form, and this probably as conjugated DDS. Partition chromatography indicated that it was not the acetylated derivative of DDS.

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17067. Fractionation of Serum Cholesterol of Chickens.*

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In a recent publication[1] we reported that only a small percentage of the total cholesterol was extracted from lyophilized normal human serum by chloroform at about 5°C. The fraction thus extracted was designated as the "readily extractable cholesterol", and extraction was found to be complete in 3 hours. This fraction is very high in the serum of nephrotic patients and hypercholesterolemic rabbits, comprising in some cases apparently all of the serum cholesterol.[1] Since atherosclerosis is a common occurrence in both of these instances, it was felt that further studies on the possible significance of this fraction would be of value.

Various authors[2-8] have shown that spontaneous arteriosclerotic lesions are common in chickens; lesions being evident in some by the time they reach 6 months of age. In the hen these lesions are found in both the elastic and muscular aorta, while they are normally confined to the muscular aorta in the rooster.[6] Since these lesions can develop while the cholesterol concentrations are less than 200 mg per 100 cc of serum,[5] it is evident that the *concentration* of cholesterol in the plasma can hardly be the chief etiological factor in the development of the arterial lesions. The purpose of the present investigation was to determine the concentration of the "readily extractable cholesterol" fraction and neutral fat of the serum of old and young chickens with the hope of obtaining some information which might help to explain atherosclerosis in the presence of relatively low concentrations of total serum cholesterol.

Experimental. Chickens of both sexes and of 3 age groups were studied. One group was 3 years of age or older, another from about 1 to 2 years and the third from 8-10 weeks old. The diet of the older birds is believed to have been mostly scraps plus some commercial feed. The young chickens received primarily starter mash. In most cases the animals were sacrificed the day they were received in the laboratory, but in a few cases, they were kept in an animal room and fed commercial laying mash for a few days prior to sacrificing. Blood for analyses was obtained by beheading the birds and collecting the blood in a beaker. One-half cc samples of the serum were lyophilized as described previously.[1] The dried serum was extracted with cold chloroform at a temperature of about 5°C for definite periods of time. The bottles were shaken every 10 to 15 minutes for the first 3 hours and then occasionally for the remainder of the extraction period. One or 2 bottles were filtered in the cold at different periods of time and the cholesterol content of the extract determined. The results shown in Tables I and II are typical of those obtained. For comparison, a number of results obtained on normal human sera are shown in Table III. The fraction designated as "neutral fat plus cholesterol" consists of neutral fat, cholesterol and cholesterol esters. The method employed for its determination has been described previously.[1]

Discussion. It will be seen from the experimental results that the "readily extractable" fraction is quite high in the serum of old hens; the older hens behaving in this re-

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[6] Dauber, D. V., *Arch. Path.*, 1944, **38**, 46.

[7] Dauber, D. V., and Katz, L. N., *Arch. Path.*, 1943, **36**, 473.

[8] Fox, H., in Cowdry, E. V.: Arteriosclerosis, New York, The Macmillan Co., 1933, Chap. 6.

TABLE I.
Extraction of Cholesterol from Lyophilized Serum of Female Chickens.

Number	Age	Cholesterol extracted in 3 hr, mg %	24 hr, mg %	72 hr, mg %	Total Chol., mg %	Neutral fat + Chol., mg %
1	>3 yrs.	225	222	—	234	1248
2	,, ,,	152	168	—	162	1207
3	,, ,,	158	171	—	169	1355
4	About 3 yrs.	200	196	—	216	2259
5	,, ,,	115	126	—	157	1487
6	,, ,,	83	82	—	115	847
7	,, ,,	34	71	—	275	419
8	About 2 yrs.	123	136	—	183	969
9	,, ,,	17	18	—	207	309
10	,, ,,	4	5	—	120	226
11	About 10 wks.	8	12	18	129	773
12	,, ,,	7	11	17	132	333
13	,, ,,	16	100	110	129	232
14	,, ,,	11	8	11	132	387

TABLE II.
Extraction of Cholesterol from Lyophilized Serum of Male Chickens.

Number	Age	Cholesterol extracted in 3 hr, mg %	24 hr, mg %	72 hr, mg %	Total Chol., mg %	Neutral fat + Chol., mg %
1	>3 yrs.	7	9	9	117	234
2	,, ,,	11	13	17	136	281
3	,, ,,	13	67	126	138	235
4	,, ,,	8	7	6	128	310
5	About 3 yrs.	7	8	10	145	202
6	About 2 yrs.	7	9	12	94	250
7	,, ,,	17	72	145	202	437
8	About 1 yr.	7	6	7	139	303
9	,, ,,	8	6	12	119	249
10	About 10 wks.	9	11	28	144	258
11	,, ,,	16	16	32	147	258
12	,, ,,	15	110	107	141	303
13	,, ,,	8	12	18	129	773
14	,, ,,	11	11	65	113	387

gard very similar to the nephrotic patient, or the hypercholesterolemic rabbit.[1] Roosters, both old and young, as well as young female chickens, showed a low value for this fraction, but in some cases the amount extracted increased with increased extraction time. We have obtained a similar increased extraction with increased extraction time in a few apparently normal human subjects as well as in a considerable number of patients. The reason for this deviation from the average normal is unknown, but preliminary experimental work with roosters indicates that it is probably dependent upon dietary composition.

It will be noted that old hens showed, on the whole, a much higher level of serum fat than did old roosters or young chickens. There also appears to be some correlation between the percentage of the cholesterol present in the "readily extractable" form and the neutral fat content of the serum, especially in the case of the old hens. Dauber and Katz[7] have emphasized the close similarity between the lesions produced by the administration of cholesterol to roosters and the spontaneous lesions in old birds, described by Fox.[8] They also have pointed out their dissimilarity from those which usually occur in roosters. The possible significance of the concentration of the "readily extractable" cholesterol as an etiological factor in the development of the lesions found in old hens and cholesterol-fed

TABLE III.
Extraction of Cholesterol of Lyophilized Normal Human Serum.

Subject	Cholesterol extracted in 3 hr, mg%	24 hr, mg %	27 hr, mg %	96 hr, mg %	Total Chol., mg %	Neutral fat + Chol., mg %
A.L.F.	20	20	25	—	227	428
D.F.M.	30	31	35	—	322	511
C.J.R.	28	25	27	—	270	428
A.F.	22	22	20	—	226	404
D.F.	22	29	28	—	240	594
A.B.	30	22	28	—	286	392
K.N.	20	26	21	23	215	533
G.W.J.	22	27	27	38	272	551
R.B.	34	38	37	35	272	735
G.C.	37	37	42	43	331	705
E.R.	27	42	55	—	267	627
L.S.	36	76	102	—	233	722
M.D.	24	24	32	—	210	365

chickens is being investigated.

Although the data which have been presented are not sufficient to enable one to draw conclusions as to the physical state in which cholesterol exists in serum, they do indicate that it probably exists in at least three states not related to the usual classification of free and ester cholesterol. These are (a) a fraction not extracted by chloroform under the conditions used; (b) a fraction which is readily extractable by chloroform, a 3 hour extraction period being adequate for its complete extraction; and (c) a fraction which is slowly extractable. We believe that (a) possibly represents cholesterol which is held firmly by the serum proteins, while (b) possibly represents cholesterol which is free in the serum or which is associated with other lipid materials such as fats and phospholipids. Our experimental evidence indicates that all the fractions contain both free and ester cholesterol.

Summary. A large percentage of the cholesterol of lyophilized serum from old hens is present in the "readily extractable" fraction. The concentration of this fraction in the serum of roosters and young chickens is quite low but, in some cases, the amount extracted increases with increased extraction time. There appears to be a correlation between the percentage of the total cholesterol present in the "readily extractable" fraction and the neutral fat content of the serum of old hens.

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17068. Acetaldehyde Disappearance and Acetoin Formation *in vitro*.*

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The presence of a diphosphothiamin enzyme in animal tissues capable of forming acetoin from pyruvate, and the utilization of acetaldehyde as a substrate in this reaction, has been described.[1] A limited study of this reaction, in brain homogenate was reported previously.[2] The present study was devoted to a comparison of the abilities of various tissues to meta-

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* Aided by a grant from the Nutrition Foundation, Inc.

[1] Green, D. E., Westerfeld, W. W., Vennesland, B., and Knox, W. E., *J. Biol. Chem.*, 1942, **145**, 69.

[2] Stotz, E., Westerfeld, W. W., and Berg, R. L., *J. Biol. Chem.*, 1944, **152**, 41.

TABLE I.
Acetaldehyde Disappearance and Acetoin Formation *in Vitro* with Rat Tissues.

		Brain		Leg muscle		Liver	
Substrate	Micromols added	AcH disapp., γ	Acetoin formed, γ	AcH disapp., γ	Acetoin formed, γ	AcH disapp., γ	Acetoin formed, γ
None	—	71*	36*	71	69	144	21
Pyruvate	3	95*	95*	124	164	142	37
Glucose	6	92†	100†	68	76	144	23
α-Ketoglutarate	6	89	67	125	124	140	0
Lactate	12	73	58	64	73	142	0
Alanine	12	75	58	69	71	141	0
Phosphoglycerate	6	70	50	96	107	143	0
Citrate	6-100	71	28	47	37	147	0

* Approximately the same results were obtained in oxygen and nitrogen.

† Approximately the same results were obtained in oxygen; in nitrogen the acetaldehyde disappearance was similar but the acetoin formation was only 46 γ.

The reproducibility of the results is indicated by the following data for 11 experiments with rat brain in the absence of substrate. Acetaldehyde disappearance: mean = 71, S.D. = 10.3, S.E. = 3.1, range = 57-88. Acetoin formed: mean = 36, S.D. = 3.55, S.E. = 1.07, range = 31-42. The effect of each substrate was compared with a simultaneous control in 2 or more experiments.

bolize acetaldehyde and form acetoin, and to the effect of added substrates upon these reactions.

Methods. To a Warburg vessel were added 1 cc of tissue homogenate in saline containing 250 mg of fresh tissue, 1.5 cc of 0.1 M phosphate buffer, pH 7.4, 20 γ diphosphothiamine in 0.1 cc water, 0.2 cc of water or substrate solution, and 0.3 cc of 0.05% acetaldehyde in the side arm. After 10 minutes equilibration at 37.5°C, the acetaldehyde was tipped in and shaken for 1 hour. A tungstic acid filtrate of the contents of the cooled flask was then prepared and analyzed for the acetaldehyde remaining[3] and the acetoin formed.[4] Mechanical and volatilization losses were small in the absence of tissue.

Results. Some of the results with rat tissues are shown in Table I. Brain and muscle breis removed acetaldehyde at about the same rate, but muscle formed twice as much acetoin as brain. Kidney brei in the absence of added substrate was similar to brain. Liver metabolized acetaldehyde at a much faster rate, but formed little acetoin. Corresponding results were previously obtained *in vivo*[5] where large amounts of acetoin were formed from acetaldehyde only when the dominant role of the liver in acetaldehyde metabolism was eliminated by hepatectomy.

The addition of pyruvate, or other substrates capable of forming pyruvate in the brei, increased both the acetaldehyde disappearance and the acetoin formation. Pyruvate and α-ketoglutarate gave large increases with both brain and muscle; glucose was also very effective in brain, but not muscle, while phosphoglycerate stimulated the reaction somewhat more in muscle than in brain. In liver, acetoin formation disappeared completely in the presence of most substrates.

Other results obtained with 6-12 micromols of substrate added to brain were: significant increases (30-60%) in acetoin formation from glycogen, lactate, alanine, phosphoglycerate and malate; minor increases (10-25%) from succinate, fumarate, glucose-l-phosphate, fructose and glycerol phosphate; no effect from butyrate, acetate, propionate, citrate, glutamate, cis-aconitate, galactose and glycerol. Anesthetization of the rat with nembutal or the addition of nembutal *in vitro* did not affect the reaction in brain tissue. The addition of 0.5 mg methylene blue to brain tissue increased the acetaldehyde disappearance 50% and the acetoin formation by 150%. When added together with pyruvate or glucose, methylene blue increased both the acetaldehyde disappearance and acetoin formation beyond that obtained with either methylene blue or the substrate alone.

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[3] Stotz, E., *J. Biol. Chem.*, 1943, **148**, 585.

[4] Westerfeld, W. W., *J. Biol. Chem.*, 1945, **161**, 495.

[5] Lubin, M., and Westerfeld, W. W., *J. Biol. Chem.*, 1945, **161**, 503.

It is reasonable to suppose that the increased acetoin formation in the presence of added substrate represented an increased amount of pyruvate available for condensation with the excess acetaldehyde. The ineffectiveness of other members of the citric acid cycle, as compared with α-ketoglutarate, the effect of glucose in brain as compared with muscle, and the comparison of glucose with fructose or glucose-l-phosphate are noteworthy considerations in any interpretation of these results.

Further studies on the disappearance of acetaldehyde in liver brei were made with 0.3 cc of 0.15% acetaldehyde in the side arm. Rat liver removed about 200 γ acetaldehyde during the incubation; added glucose had no effect. Small increases (10%) in the amount removed resulted from the addition of pyruvate, lactate or alanine. 100 μM of citrate or 0.5 mg methylene blue increased the acetaldehyde disappearance by 25% and 40%. Dog liver gave similar results except that methylene blue had no effect. The methylene blue effect in rat liver as contrasted with dog liver is possibly related to the presence of xanthine oxidase in rat liver only; methylene blue stimulates aerobic oxidation by xanthine oxidase.

Summary. In agreement with previous ***in vivo*** studies, liver homogenate metabolized acetaldehyde more rapidly than other tissues and without the formation of much acetoin. Methylene blue stimulated the aerobic metabolism of acetaldehyde in rat liver but not dog liver.

Skeletal muscle formed more acetoin from acetaldehyde than did brain or kidney. Pyruvate, α-ketoglutarate and phosphoglycerate increased the acetoin formation from acetaldehyde by muscle; pyruvate, α-ketoglutarate and glucose were effective stimulators of acetoin formation in brain.

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17069. Isolation of an L Type Culture from a Gram Positive Spore-Bearing Bacillus.*

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During the last 10 years L type cultures have been isolated from many Gram negative bacilli. The author tried repeatedly to apply to Gram positive bacilli the methods which proved successful in Gram negative species. Success was obtained first in the summer of 1948 with a Gram positive bacillus isolated from a contaminated blood culture. The broth cultures of this bacillus were pleomorphic. After 24 - 48 hours of incubation almost all bacilli swelled to round bodies as in the cultures of some Bacteroides strains. This pleomorphic culture transferred to media of various compositions produced only bacterial growth, but when a penicillin cup was placed on soft horse-serum agar plates and the plates were incubated anaerobically, a few microscopical L type colonies developed near the edge of the zone of inhibition. Growth of these tiny colonies in pure culture was obtained by cutting out a block of agar containing the colonies and transferring it to horse serum agar containing 400 units of penicillin per cc. The colonies grew without difficulty in subsequent subcultures.

Following this observation several samples of dust and earth were planted on similar plates containing varying concentrations of

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penicillin. L type cultures were not observed in any of these plates. However, from one earth sample a large Gram positive bacillus was isolated which, when transplanted on penicillin plates, produced pleomorphic forms and tiny L type colonies. Neither the first nor the second Gram positive bacillus produced spores.

These Gram positive bacilli probably originated from the soil in which pleuropneumonia-like organisms are often present. Thus a contamination of the cultures with such organisms must be considered. Usually it is difficult to exclude this possibility and to prove that the L form grow from the bacilli. The needed proof was obtained with a third Gram positive bacillus. This bacillus produced spores and the L form grew in the same manner from the heated spores as from the original culture. This observation, while it excludes an accidental contamination of the cultures, does not prove in itself that the L cultures represent a changed growth form of the bacillus. They could be parasites of the bacilli and Klieneberger,[1] for example, even at present prefers this hypothesis. In the opinion of the author the evidence in the case of Gram negative bacilli[2–4] supports the conception that the L forms and the bacilli are growth forms of the same organism.

The third Gram positive rod was obtained from a thioglycollate broth inoculated from a wound. Growth in the first subculture developed only anaerobically, and on further subcultures also anaerobic growth was more abundant than aerobic. The colonies on blood agar plate incubated aerobically were flat and slightly irregular, and after a few days the culture spread as a thin film on the surface of the medium. Large elongated spores, usually at the ends of the rods, developed abundantly. A distinguishing property of the bacillus was its tendency to swell into large pleomorphic forms. Some of the bacterial threads spreading on blood agar plate autolyzed and disintegrated and the fragments swelled into small and large round forms. In thioglycollate broth some of the rods grew to large size and became markedly swollen around the developing spores. This tendency to form round bodies is rarely observed in Gram positive rods. Exact identification of saprophytic strains is difficult and has not been attempted. However, the development of spreading growth on the surface of the agar under aerobic conditions and the formation of acid without gas in various sugars makes it probable that it belongs to the genus *Bacillus*.

A thioglycollate broth culture of this strain was planted on soft agar plates containing 10% horse serum and 400 units of penicillin per cc. The plates were made anaerobic using Fortner's technic.[5] Use of a similar medium permitted isolation of L type colonies from various Gram negative bacilli.[3,4] The development of the third strain on this medium was similar to that of Gram negative bacilli which produced L type colonies. Many bacilli swelled within a few hours into large flat round bodies. From these tiny colonies developed which grew best in transplants on soft nutrient agar plates without serum. The tiny colonies on the original penicillin plates and in the subcultures are illustrated in the photographs. Their appearance and morphology were similar to the L_1 and to the other L type colonies obtained from Gram negative bacilli. The colonies grew into the medium and consisted of soft fragile cells which were often very small and had the tendency to swell into large bodies.

In order to determine whether L cultures can be obtained from heated spores, well-sporulated broth cultures were sealed in thin glass tubes and immersed in water at 85°C for 10 minutes. (Previous experiments had shown that the spores were killed in boiling water). The heated spores when transferred to penicillin plates produced no growth. Broth cultures of bacilli obtained from the heated spores produced L type colonies on penicillin plates as the original cultures.

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[4] Dienes, L., *J. Bacteriology*, 1949, in press.

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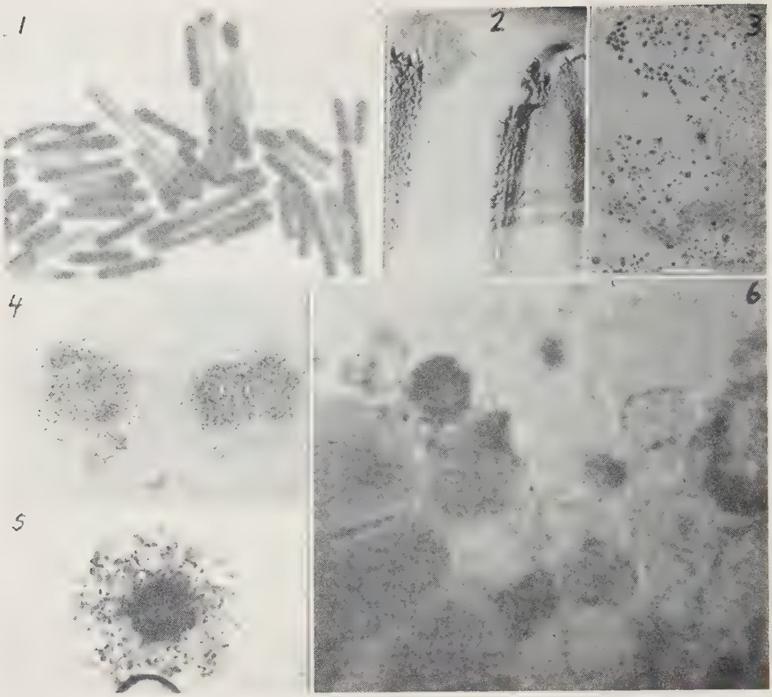

Fig. 1.

1. Bacilli from a broth culture. Slightly stained with crystal violet. (× 2000.)
2. Culture with spreading growth on aerobic blood agar plate. (× 2.)
3. Tiny colonies on penicillin plate after 5 days' incubation. (× 2.)
4. Tiny colonies from a subculture on a soft nutrient agar plate. Unstained. (× 100.)
5. A colony similar to those in Photograph 4 stained with methylene blue on the surface of the agar. (× 100.)
6. Large bodies in the periphery of a tiny colony stained with methylene blue. (× 2000.)

It was interesting to observe the development of the cultures when a soft agar plate was inoculated with heated spores and 100-500 units of penicillin was deposited in a small trough made in the agar. The plates were incubated anaerobically. No growth developed in a wide area around the penicillin trough. The penicillin diffused in this area before the spores germinated. Many tiny colonies developed in the adjoining zone. Here the spores germinated and the young vegetative cells were exposed to the diffusing penicillin. The tiny colonies consisted of large round forms produced by swelling of the vegetative cells and many L colonies began to grow from these large forms. Further away from the penicillin trough the usual bacillary colonies developed. The observations reported indicate that L forms were not cultivated directly from the heated cultures. They apparently survive with the bacteria and develop under appropriate conditions from the vegetative cells. The L strains isolated from the second and third bacilli were tested for heat resistance. Heating of the cultures for 15 minutes at 60°C destroyed their viability.

It is of interest also that descendants of single bacillary colonies varied considerably in their tendency to produce L type colonies and in the viability of these colonies. In one experiment 4 colonies were picked from a culture of bacillus No. 3 on a blood agar plate. Marked differences persisting in subculture were present among the descendants of these colonies. The descendant of one produced more L type colonies than the others and the

L colonies grew easier in subculture. Among the descendants of the colony which was least susceptible to the influence of penicillin, only a few bacilli swelled to large form when their growth was inhibited by penicillin and only a few L type colonies were produced. A similar individual behavior of different strains belonging to the same species has been noted in all species which produce L colonies, but the differences are usually apparent only between strains of different isolation.

The L type cultures obtained from Gram positive bacilli differ from each other and from those obtained from Gram negative bacilli in many respects, although their fundamental morphology and structure of the colonies conform to those of the pleuropneumonia group. The colonies obtained from the two bacilli mentioned first remained very small (10-20 μ) on the original plates and in the early transfers stained poorly with methylene blue. After several transfers they grew more abundantly. They grew equally well with or without horse serum in the medium. The hardness of the agar played an important role in their growth. In the absence of horse serum growth developed only on hard agar plates containing 1.5%-2% of agar. If the agar was softened by the addition of equal amounts of broth, it did not support their growth. Soluble starch markedly improved growth, while glucose, maltose, sucrose and penicillin exerted no influence. The colonies continued to increase in size for a period of two weeks. No growth could be obtained in nutrient or thioglycollate broth, although the colonies transferred with a block of agar into the broth continued to increase in size on the agar. In morphology these cultures were quite similar to the filterable strains isolated from sewage by Laidlow and Elford.[6] The cultures consist of very small (0.3-0.5 μ), fragile coccoid and bacillary forms and they swelled only to moderate size on the surface of the colonies. The strains of Laidlow and Elford grow abundantly in broth and aerobically on nutrient agar. In this respect they differ considerably from ours.

Young colonies of the L strain isolated from the third spore-bearing bacillus were more similar in appearance to the L cultures isolated from *Streptobacillus moniliformis* and Bacteroides and consisted of similar organisms. If the colonies were well spaced, they increased in size for several weeks extending as an opaque mass below the surface of the agar reaching the size of 1-2 mm. They grew best on nutrient agar in the absence of horse serum. In contrast to the other two strains growth developed only on soft agar plates and was inhibited by the usual concentration of agar. Sucrose seemed to favor their growth but its effect was not marked; starch exerted no influence. No growth was obtained in nutrient or thioglycollate broth. The original bacillary forms have not been recovered thus far from any of the three L cultures isolated from Gram positive bacilli.

Summary. Colonies similar in appearance and properties to the L type colonies of Gram negative bacilli were isolated from cultures of several large Gram positive bacilli. The L type colonies obtained from a spore-producing strain grew equally well from cultures obtained from heated spores as from the original culture, indicating that the L type colonies grew from the bacilli and were not an accidental contamination of the culture.

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17070. Effect of Folic Acid, Liver Extract, and Vitamin B_{12} on Hemoglobin Regeneration in Chicks.*

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No change in the blood hemoglobin level has been found to accompany the growth response of chicks to the injection of antipernicious anemia (APA) liver extracts[1] or vitamin B_{12}[2] when fed a corn-soybean oil meal basal ration containing adequate folic acid. The importance of folic acid for hemoglobin regeneration has been demonstrated with experimental anemias in several different species showing anemia and in the therapy of human macrocytic anemias. APA liver extracts have been found to be ineffective for the cure of anemia induced by folic acid deficiency in the chick[3,4] and in the rat.[5] Data presented here, based upon the rate of hemoglobin regeneration after production of a severe, chemically-induced anemia in folic acid depleted chicks, show that liver extracts or vitamin B_{12} are ineffective in increasing the rate of hemoglobin formation when given alone, but show a stimulatory effect when given with folic acid.

Experimental. Straight-run (New Hampshire ♂♂ x Single Comb White Leghorn ♀♀) crossbred chicks which were the progeny of hens fed diet B-1 described previously[6] were used in all studies. The chicks were housed in electrically heated batteries with raised screen floors. Feed and water were supplied *ad libitum*. The chicks were wing-banded when one day old. Weights were recorded at weekly intervals during the depletion period.

All chicks received a folic acid deficient purified ration containing dextrin 61, alcohol-extracted casein 18, gelatin 10, salts V 6,[7] soy oil 5, 1-cystine 0.3, and fortified haliver oil (60,000 U.S.P. units of vitamin A, 6,000 A.O.A.C. units of vitamin D_3 per g) .04 g; thiamin 0.3, riboflavin 0.6, nicotinic acid 5.0, pyridoxine 0.4, calcium pantothenate 2.0, biotin 0.02, choline chloride 150, inositol 100, 2-methyl-1,4-naphthoquinone 0.05 and α-tocopherol 0.3 mg.

Chicks fed the folic acid deficient, purified ration developed a slight anemia in 14 to 24 days. Hemoglobin levels varied widely, but the decrease averaged 1 to 2 g below normal. At the end of this depletion period a single intramuscular injection of phenylhydrazine hydrochloride (2.0 mg per 100 g body weight) was given. Severe anemia developed within 2 days (Hb range, 1-5 g %). Reagent grade phenylhydrazine hydrochloride was treated with charcoal and recrystallized from hydrochloric acid solution. An aqueous solution (10 mg per cc) was injected into the pectoral muscle using a 1 cc calibrated hypodermic syringe.

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We are indebted to Merck and Co., Inc., Rahway, N. J., for pure vitamin B_{12} and for crystalline vitamins; to the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for pteroylglutamic acid and pteroyltriglutamic acid; to Parke, Davis and Co., Detroit, Mich., for pteroylheptaglutamic acid.

† Present address: Department of Biochemistry, University of Alberta, Edmonton, Canada.

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Comparable groups of chicks having hemoglobin values ranging from 2 to 4 g % were then arranged. Experimental treatments were followed by determining hemoglobin levels at 2 to 4 day intervals, using the alkaline oxyhemoglobin method[8] as adapted for the Evelyn colorimeter.[9] Blood samples were taken directly by puncture of a wing vein.

Dosages of phenylhydrazine hydrochloride from 1 to 6 mg per 100 g body weight were tested. 2.0 mg was selected as the smallest dosage which caused a suitable decrease in hemoglobin level within 2 days. It was also observed that a rapid return to normal hemoglobin level occurred if the ration contained adequate folic acid. The hemoglobin level of normal chicks dropped to about 5 g % within 2 days after the injection of phenylhydrazine hydrochloride. Three to 4 days following this anemic condition, their hemoglobin level had returned to normal. Consequently, small amounts of folic acid must be given to the folic acid depleted, anemic birds to prolong the period of recovery so that differences in rate of hemoglobin regeneration can be measured.

Experiment 1. Four groups of deficient chicks, having hemoglobin values of 2 to 4 g %, were selected on the 16th day (2 days following the injection of 2.0 mg of phenylhydrazine hydrochloride per 100 g body weight). Six chicks were used in each group and no losses occurred during the following treatments (1) control group, untreated, (2) liver extract (Lilly, reticulogen, 20 U.S.P. units per cc), 1 unit per bird, injected, (3) folic acid, 20 γ per bird, injected, (4) liver extract, 1 unit per bird and folic acid, 20 γ per bird, injected. The dosages were given on the 16th, 18th and 20th days.

The changes in the hemoglobin values following the above treatments are shown in Fig. 1. Liver extract alone did not influence the rate of hemoglobin regeneration. By the 24th day, the hemoglobin values of the groups receiving folic acid and folic acid in combination with liver extract had returned to normal and did not differ significantly from each other. The least significant difference between treatments on the 24th day was 1.23 g %.

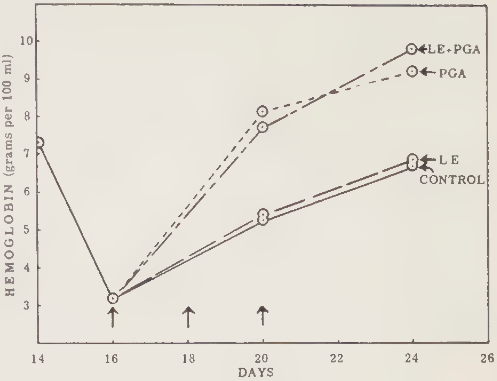

Fig. 1.
Rate of Hemoglobin Regeneration in Anemic Chicks.

The control group was untreated. In the other groups each bird received: LE—1 unit liver extract; PGA—20 γ folic acid; LE + PGA—1 unit liver extract and 20 γ folic acid, injected on the 16th, 18th, and 20th days.

Exp. 2 The pattern of Exp. 2 was similar to that used in Exp. 1 except that the depletion period was extended to 21 days before injection of phenylhydrazine hydrochloride. Pteroylheptaglutamic acid and pteroyltriglutamic acid were tested at equimolar dosage to pteroylglutamic acid (PGA). All of the 5 chicks used in each test group survived.

As shown in Fig. 2, liver extract alone did not increase the rate of hemoglobin regeneration. PGA alone did. The triglutamate and heptaglutamate forms were as active as PGA. Liver extract with folic acid resulted in more rapid regeneration of hemoglobin than was observed on folic acid alone. A significant difference in the average hemoglobin values of these two groups was noted on the 29th day and again on the 31st and 33rd days. The least significant difference between treatments by the 33rd day was 1.27 g %.

Exp. 3. Chicks were depleted for 24 days on a folic acid deficient, purified ration in which sucrose was used as the source of carbohydrate. In addition to the treatments used in Exp. 1 and 2, pure vitamin B_{12} was tested alone and in combination with folic acid. In the control group, 5 of the 8 birds

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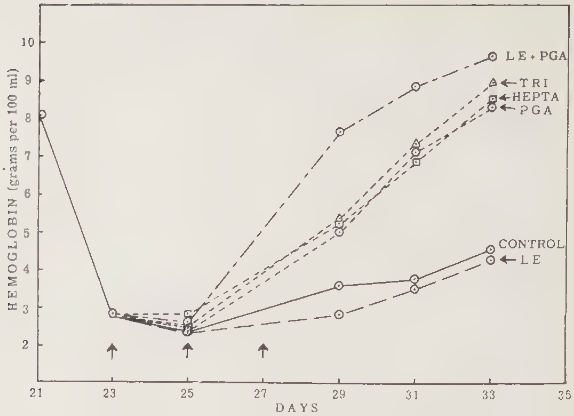

FIG. 2.

Rate of Hemoglobin Regeneration in Anemic Chicks.

The control group was untreated. In the other groups each bird received: LE—1 unit liver extract injected on the 23rd, 25th, and 27th days; HEPTA—pteroylheptaglutamic acid and TRI—pteroyltriglutamic acid at equimolar dosage to PGA—pteroylglutamic acid, 5 γ, 10 γ, and 20 γ injected on the 23rd, 25th, and 27th days respectively; LE + PGA—1 unit liver extract and 5 γ folic acid injected on the 23rd day, 1 unit liver extract and 10 γ folic acid injected on the 25th day, 1 unit liver extract and 20 γ folic acid injected on the 27th day.

survived on the 38th day and 3 survived beyond the 42nd day. At least 7 of the 8 chicks survived beyond the 42nd day in each of the other test groups.

The average hemoglobin values for each group are shown in Fig. 3. The pattern of hemoglobin regeneration induced by vitamin B_{12} was the same as that observed with liver extract. Vitamin B_{12} alone had no greater effect than liver extract, yet in combination with folic acid the rate of hemoglobin regeneration was more rapid than that resulting from treatment with folic acid alone. By the 42nd day, the least significant difference between treatments was 1.20 g % hemoglobin.

In addition to the group shown in Fig. 3 which received 1 γ of vitamin B_{12} plus 10 γ folic acid on the 26th and 29th days, a second group of 8 chicks which received 0.1 γ of vitamin B_{12} plus 10 γ of folic acid on these days showed an average hemoglobin value of 8.24 g % on the 42nd day and another group which received double the dosage of folic acid alone (20 γ) on these days had an average hemoglobin value of 7.71 g % on the 42nd day.

Discussion. A series of 5 experiments in all were carried out, each of which included the 4 experimental treatments outlined in Exp. 1. Liver extracts used in the treatment of pernicious anemia were not effective in stimulating the rate of hemoglobin regeneration in anemic chicks depleted of folic acid. In each of the 4 experiments having a depletion period of 21 days or longer, the rapid rate

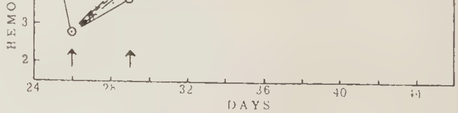

FIG. 3.

Rate of Hemoglobin Regeneration in Anemic Chicks.

The control group was untreated. In the other groups each bird received: LE—1 unit liver extract; B_{12}—1 γ vitamin B_{12}; PGA—10 γ folic acid; LE + PGA—1 unit liver extract and 10 γ folic acid; B_{12} + PGA—1 γ vitamin B_{12} and 10 γ folic acid, injected on the 26th and 29th days.

of hemoglobin regeneration following treatment with liver extract and folic acid was significantly greater than the rate observed following treatment with an equal dosage of folic acid alone. Less than 1 γ of folic acid was present in the 1 U.S.P. unit of liver extract injected. The component causing the stimulation of hemoglobin regeneration in the presence of folic acid appears to be vitamin B_{12}.

The metabolic interrelationship between folic acid and vitamin B_{12} is still obscure. Since in the anemic chicks, folic acid alone in large amounts will bring about rapid and complete return to normal hemoglobin level, the observed stimulation of hemoglobin regeneration may be due to an indirect action of vitamin B_{12} which makes more folic acid available to the animal organism. Vitamin B_{12} may also act directly in the normal mechanism of hemoglobin formation. This is implied by the activity of vitamin B_{12} in cases of pernicious anemia. The chicks used in these studies, like those used for the growth assay of vitamin B_{12},[2] were depleted by control of the hen's diet. Under the special conditions described, the stimulation of hemoglobin regeneration may be a demonstration of the direct role of vitamin B_{12} in the mechanism of hemoglobin formation. This work emphasizes that folic acid and vitamin B_{12} may have distinct and separate functions and indicates that vitamin B_{12} does not have optimum activity unless folic acid is present in adequate amounts.

The effectiveness of the technic is demonstrated by the similar response to the three forms of folic acid shown in Fig. 2. In each experiment, analysis of variance was applied on the last day. This is a more severe test of significance, since individual variation becomes greater as the interval following treatment increases. For example, in experiment 2 the least significant difference between treatments on the 31st day was 1.05 g % hemoglobin. By the 33rd day the least significant difference had increased to 1.27 g %.

Phenylhydrazine hydrochloride is used in the therapy of polycythemia vera. The anemia induced is due to hemolysis of the mature red blood cells, but the mechanism is not known.[10] Yeshoda and Damodaran[11] used a phenylhydrazine induced anemia in rats in a study of the influence of tryptophan on hemoglobin formation. The application of this technic to folic acid depleted chicks increases the range over which differences in hemoglobin levels can be measured and permits selection within narrow limits of comparable groups of chicks having a severe anemia.

Summary. A severe anemia was induced in chicks by the intramuscular injection of phenylhydrazine hydrochloride (2.0 mg per 100 g body weight) following a depletion period on a folic acid deficient purified ration. Chicks were selected within narrow limits (2-4 g % Hb) and arranged into comparable groups to study the effect of folic acid, liver extract and vitamin B_{12} on the rate of hemoglobin regeneration.

Liver extract alone did not influence the rate of hemoglobin formation. When the depletion period was 21 days or longer, the combination of liver extract and folic acid caused a more rapid regeneration of hemoglobin than treatment with a similar dosage of folic acid alone. Pure vitamin B_{12} completely replaced liver extract in stimulating the formation of hemoglobin in the presence of folic acid.

The authors express appreciation to Dr. W. W. Cravens, Poultry Department, University of Wisconsin, and Dr. A. R. Robblee, Poultry Department, University of Alberta, for their cooperation.

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[10] Sollmann, T., A Manual of Pharmacology, W. B. Saunders Co., Philadelphia and London, 1942, 6th ed., 666.

[11] Yeshoda, K. M., and Damodaran, M., *Biochem J.*, 1947, **41**, 382.

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17071. Influence of Chronic Morphine Poisoning on Growth and Metabolism of the Albino Rat.*

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The appearance of signs of hyperirritability following withdrawal of morphine in certain animal species which have received the drug for a prolonged period of time is fairly well established. Plant and Slaughter[1] and Barbour, Porter and Seelye[2] have presented evidence which might suggest that in the dog these phenomena are related to an increased basal metabolic rate. In line with a conception relating withdrawal signs to an elevated metabolism of tissues is the observation by Shideman and Seevers[3] that skeletal muscle obtained from rats chronically poisoned with morphine exhibits an increased oxygen consumption which attains a maximum value 48 hours following withdrawal of the drug. At this time oxygen uptake is approximately twice that of muscle obtained from normal animals. The presence of such a marked increase in the metabolism of skeletal muscle *in vitro* should be reflected in an elevated basal metabolic rate of the animal during the withdrawal period. On the other hand, the presence of certain factors existing *in vivo* (neural and humoral) might mask this increased activity which would become apparent only when relieved of these factors. Swann[4] stated that, during addiction in the rat, morphine has a specific dynamic action on metabolism but that withdrawal is followed by a violent depression of the B.M.R. In order to obtain more information on the metabolic effects of addiction to morphine and its withdrawal in the rat, the following experiments were performed.

Experimental. Two groups of albino rats (12 in each group), averaging approximately 100 g in weight were placed on a diet of ground Purina chow for a period of 9 days. During this period individual food intake, and weight change were determined. The B.M.R. for each animal was obtained by measuring respiratory exchange over two 30 minute periods in a closed system maintained at 29°C in a constant temperature water bath. Air, to which oxygen was added as required, was continually circulated through the system. Oxygen consumption was measured by means of a small spirometer and carbon dioxide was absorbed in alkali which was analyzed for carbonate before and after each experimental period. Surface area for each animal was calculated by the formula of Diack.[5]

Following this control period one group of animals was chronically poisoned with morphine as described previously.[6] When the dose of morphine had reached 200 mg/kg (over a period of 8 weeks) and been maintained at that level for at least a week the drug was discontinued and the B.M.R. again determined on each rat in this group as well as in the control groups. During the fourth week of the experiment, one of the animals receiving morphine died and any previous results obtained on that animal were not used in determining means and in statistical analysis.

Results. At the beginning of the experiment the mean weight of each group of animals was approximately the same (Fig.1). During the control period, prior to the administration of morphine, the average gain in

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* This investigation was supported by a Research Grant (M. H. Seevers) from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

[1] Plant, O. H., and Slaughter, D. H., *J. Pharm. and Exp. Therap.*, 1936, **58**, 417.

[2] Barbour, H. G., Porter, J. A., and Seelye, J. M., *J. Pharm. and Exp. Therap.*, 1939, **65**, 332.

[3] Shideman, F. E., and Seevers, M. H., *J. Pharm. and Exp. Therap.*, 1942, **74**, 88.

[4] Swann, H. G., *Am. J. Physiol.*, 1941, **133**, 467.

[5] Diack, S. L., *J. Nutrition*, 1930-31, **3**, 289.

[6] Shideman, F. E., and Seevers, M. H., *J. Pharm. and Exp. Therap.*, 1941, **71**, 383.

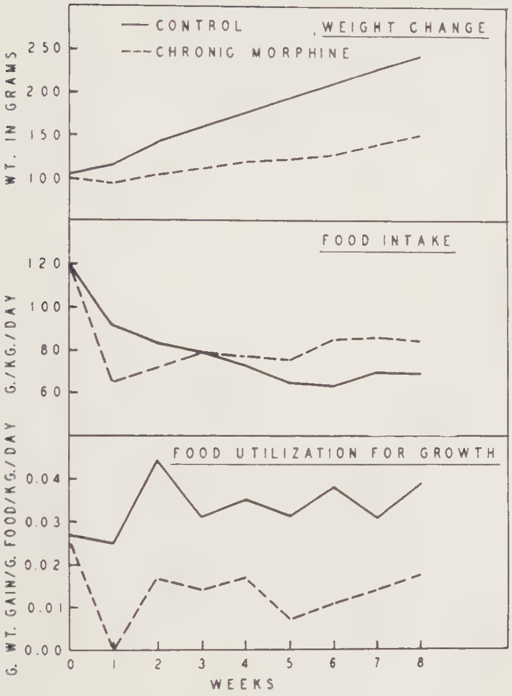

Fig. 1.

The effect of chronic morphine poisoning on the growth, food intake, and food utilization of the albino rat.

weight, food intake in g/kg and grams of weight gain per gram of food consumed per kilo of body weight per day were about equal in the two groups. (Fig. 1). This latter figure gives some indication of the utilization of food for synthesis of body tissues. During the period of the experiment, the rats receiving morphine showed a slight loss in weight during the first week followed by a gradual increase during the ensuing seven weeks. The rate of growth was much slower than in the control group. This great difference in rate of weight gain is out of proportion to the difference in food intake in the two groups (Fig. 1). The food intake of the group of animals receiving morphine is somewhat less than that of the other group of animals during the first 2 weeks but after this time is equal to, or greater than that of the control animals. The normal animals consistently utilized much more food for growth than did the animals receiving morphine. This is shown by the values obtained for grams of weight gain per day per gram of food consumed per kilogram of body weight.

Before administration of morphine was begun in the one group of rats, the mean basal metabolic rates in the two groups were approximately the same (Table I) and there was no statistically significant difference between the 2 (t of dif. = 0.664). At the end of 8 weeks the values in both groups dropped significantly. However, the fall in the mean B.M.R. of the chronically morphinized rats at the 48 hour withdrawal period was only 47.50% as great as that obtained in the control group and was significantly higher (t of dif. = 3.824) than that of the normal animals at that time. It will also be noted that whereas the mean R.Q. of the normal rats decreased slightly over the 8 weeks period, that of the chronically morphinized rats fell more markedly and significantly (t of dif. = 3.41).

Discussion. There is little information in the literature in regard to the effects of chronic morphine poisoning and withdrawal of the drug on weight change, food utilization and metabolic rate of the rat. Myers and Flynn[7] administered 1 to 5 mg/kg/day to young growing rats over a period of 55 days, with only a slight impairment of growth. If higher doses had been given, it is probable more pronounced effects might have been obtained. Sollmann[8] had previously reported that feeding morphine in the diet in doses increasing from 0.01 to 35 mg/kg/day over a period of 21 weeks increased the rate of growth of young rats during the first 15 weeks. The significance of such results is questionable since there were only two animals in the experimental group. Hildebrandt[9] poisoned rats with morphine, beginning with a dose of 100 mg/kg/day and increasing rapidly to 600 to 1000 mg/kg/day. After two weeks his experimental animals showed a marked weight loss. It is surprising, in view of the fact that he

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[7] Myers, H. B., and Flynn, J. B., Proc. Soc. Exp. Biol. and Med., 1928, **25**, 786.

[8] Sollmann, T., *J. Pharm. and Exp. Therap.*, 1924, **23**, 449.

[9] Hildebrandt, F., *Arch. Exp. Path. Pharmakol.*, 1932, **92**, 68.

TABLE I.
Effect of Withdrawal Following Chronic Morphine Poisoning on the Respiratory Quotient and Metabolic Rate of the Rat.

	No. of animals	Initial		Final	
		BMR	R.Q.	BMR	R.Q.
Normal	12	51.5 ($t = 55.6$)	.829 ($t = 53.9$)	41.95 ($t = 29.4$)	.812 ($t = 36.2$)
Chronic Morphine	11	53.3 ($t = 29.2$)	.830 ($t = 48.08$)	48.86* ($t = 44.1$)	.764* ($t = 94.8$)

* Values obtained 48 hours after withdrawal of the drug which had been administered in increasing doses (from 20 mg/kg/day to 200 mg/kg/day) over a period of 8 weeks.

was using larger doses and younger rats than were employed in these experiments, that the inhibition of growth was not noted sooner.

It is difficult to interpret the changes in basal metabolic rate of the two groups of animals in the experiments reported here. If the final values were the only ones available to compare, it could be concluded that during the withdrawal period there was an increase in metabolism. However, in view of the fact that the metabolic rate of both groups decreased and since the decrease in each is roughly proportional to the gain in weight, it is possible the final difference is merely due to a difference in weight between the two groups. Nevertheless, the lower respiratory quotient and decreased utilization of food for body growth might indicate an increase in metabolic rate which is not apparent from the values obtained for basal metabolic rates. Further experiments are planned to clarify the questions provoked by these results.

Summary. Chronic administration of morphine in doses increasing from 20 mg/kg/day to 200 mg/kg/day over a period of 8 weeks to young albino rats resulted in a marked suppression of growth. This occurred in spite of a greater food intake per unit of body weight. In addition, the animals receiving morphine demonstrated a significant reduction in their respiratory quotients at the 48 hour withdrawal period. The interpretation of the changes in metabolic rate is not apparent on the basis of the results obtained.

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17072. Value of Calcium Hypophosphite and Other Calcium Compounds as Calcium Supplements in Calcium-Low Diets.

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The value of hypophosphites as therapeutic agents has never been objectively demonstrated and Marriott's criticism of their use[1] has largely been accepted. However, calcium hypophosphite is well worth while being considered as a means of convenient calcium administration, where such therapy is desired, because it is high in this element (23%), does not have the objectionable taste of most calcium salts and, in contrast to gluconate, is adequately soluble. The hypophosphite ion, as a stable acid, balances the alkalinity of the calcium, which effect persists even if there should occur a transformation into phosphoric acid. Therefore, the salt does not shift the acid-base equilibrium to the alkaline side as is the case with salts of carbonate producing acids. The original purpose of the present investigation was a comparison of calcium hypophosphite with dicalcium phosphate and calcium gluconate as a supplement in a calcium-poor diet. According to Marriot's report the hypophosphite anion is largely excreted unchanged; for that reason, the hypophosphite P was regarded as non-available in our first tests. When the early observations suggested that this might not be the case, a study of the influence of readily available P and hypophosphite P at varying Ca/P ratios was included.

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[1] Marriot, McKim, *J. Am. Med. Assn.*, 1916, **76**, 486.

Experimental. Groups of 7 to 8 Sprague-Dawley rats were placed on a diet prepared according to Sobel, Rockenmacher and Kramer,[2] which consists of 70 parts yellow corn meal, 16 wheat gluten, 10 brewer's yeast, and 1 NaCl with the addition of cod liver oil to supply 0.1 unit vitamin D per gram. Analysis showed a content of 0.03% Ca and 0.33% P, which is in accord with the figures given by these authors. All animals were kept for 14 days on this diet as a depletion period, and the control group for an additional 25 days. Additions of calcium hypophosphite, gluconate and secondary phosphate were used in the first comparative tests (A to E in table). Nucleic acid was used in some groups to equalize the content in available phosphorus without adding a phosphate. The additions in groups F to O served to vary the Ca/P ratios as widely as the P content in the basic diet would permit, with proper distinction of P, known to be utilized, and hypophosphite P.

The rats at the beginning of the depletion period were of an age specified for vitamin D assay in the U.S.P. It turned out that the range of permissible variations in weight and age, as set for that test, was too wide than was best suited for these experiments. The rats of the last group were apparently a few days older (Nos. A_3 to O), and consequently had larger calcium reserves. For that reason, each group can be compared only with its own control group. The animals were weighed at the beginning and end of the 25 days' test period, then killed and the femora dissected. The bones, dried at 80°, were freed of all adhering tissue, weighed, incinerated and the ash determined.

Results. It is evident that the assimilation of calcium is about the same whether supplied as hypophosphite, gluconate, phosphate or carbonate. While 130 mg Ca per 100 g diet is inadequate for maximal calcification of the bones, 530 mg gave results equal to 730 and 1030 mg as shown in Groups I to O. The final body weight at the end of the test period was within the same range in all groups disregarding the calcium intake, with exception of group A_1. While it is possible that the smaller animals were more sensitive to Ca deficiency, it must be pointed out that group B, with equally low Ca feeding, showed only a slight depression in weight. The weight and ash content of the femora reflect more definitely any calcium deficiency than the body weight.

The experiments on Ca/P ratio were limited by the 330 mg P content in the basic diet, which was found to be high enough to prevent any acute P deficiency. As a consequence the results appear to be exclusively a function of the Ca supply. A ratio of Ca/P of 1:2.54 (Group F) was positively not detrimental to bone formation, and neither was one of 1:4.9, (Group H) assuming that hypophosphite P is utilized. This, however, we were unable to prove because of the impossibility to produce a P deficiency with this diet and of the latitude in tolerated Ca/P ratios.

Shohl[3] has claimed that a low Ca/high P ratio lowers the ash content in the bones of the rats. He used, indeed, a ratio of 1:16, but only by sacrificing the absolute Ca content in the diet. When 0.12% Ca was given with 2% P the ash was 36%, but with 0.12% Ca and 0.5% P the ash was the same. This is in keeping with our conclusion that the quantity of Ca and not the Ca/P ratio is the limiting factor. Since, according to Shohl's statement, excessive quantities of phosphorus are toxic, it is difficult to prove a detrimental effect of a low Ca/P ratio at an adequate Ca intake.

Some of the ulnae and radii of the A_1 to E groups were examined by the Ag line test as used in vitamin D assay, but no differences were detected.

It seems that bone formation is not the most

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[2] Sobel, A. E., Rockenmacher, M., and Kramer, B., *J. Biol. Chem.*, 1945, **158**, 475.

[3] Shohl, Alfred, *J. Nutr.*, 1936, **11**, 275.

TABLE I.

	Addition to basic diet	Mg Ca per 100 g	Mg P per 100 g	Ratio, Ca/P	Wt. at		Femora wt., mg	Ratio femora mg to wt. g	% ash in femora
					beginning of test period	end			
A_1		30	330	1:11	68.0	91.0	301.5	3.13	31.24
					±7.6	±10.8	±22.5	±0.51	±1.71
B	Nucleic acid	30	330	1:13.6	72.5	110.1	320.5	2.91	31.45
			77		±10.1	±14.5	±16.4	±0.30	±0.68
C	$CaHPO_4$	30	330	1:3.13	60.7	122.8	352.7	2.87	41.73
	$2H_2O$	100	77		±3.2	±8.8	±14.6	±0.13	±1.74
D	$Ca(H_2PO_2)_2$	30	330	1:3.13	64.0	134.9	395.6	2.93	40.76
	Nucleic acid	100	77		±3.0	±4.4	±18.8	±0.15	±1.08
			(155)	(1:4.3)					
E	Ca gluconate	30	330	1:3.13	61.1	115.6	378.7	3.28	41.86
	Nucleic acid	100	77		±3.8	±9.0	±23.0	±0.18	±1.53
A_2		30	330	1:11	83.9	131.7	268.4	2.04	30.70
					±7.8	±7.9	±16.5	±0.08	±0.40
F	$Ca(H_2PO_2)_2$	30	330	1:2.54	83.2	135.1	341.5	2.54	39.22
		100	(155)	(1:3.73)	±9.0	±13.8	±27.5	±0.17	±1.27
G	Ca gluconate	30	330	1:2.54	88.4	135.9	329.3	2.42	39.06
		100			±10.8	±9.8	±32.9	±0.19	±1.01
H	$Ca(H_2PO_2)_2$	30	330	1:2.54	83.5	131.4	324.6	2.48	40.49
	$Na(H_2PO_2)$	100	(155)	(1:4.9)	±3.1	±9.2	±17.2	±0.12	±1.29
	H_2O		(155)						
A_3		30	330	1:11	95.2	129.3	231.2	2.56	36.9
					±7.7	±10.7	±39.7	±0.23	±1.2
I	$Ca(H_2PO_2)_2$	30	330	1:0.32	96.0	121.2	472.6	3.93	52.5
		1000	(1550)	(1:1.8)	±8.4	±10.7	±45.1	±0.12	±1.2
K	Ca gluconate	30	330	1:0.32	97.2	128.8	466.9	3.65	51.6
		1000			±5.0	±19.1	±59.4	±0.29	±2.0
L	Nucleic acid	30	330	1:0.61	96.7	125.2	476.6	3.82	53.0
	$Ca(H_2PO_2)_2$	1000	300		±6.4	±9.3	±24.6	±0.24	±0.8
			(1550)	(1:2.0)					
M	Nucleic acid	30	330	1:0.9	96.6	125.3	458.9	3.66	52.4
	$Ca(H_2PO_2)_2$	700	300		±2.8	±11.3	±35.7	±0.18	±1.1
			(1085)	(1:2.4)					
N	$Ca(H_2PO_2)_2$	30	330	1:0.62	101.4	140.3	504.2	3.60	53.2
		500	(775)	(1:2.09)	±7.6	±13.6	±44.9	±0.24	±1.6
O	$CaCO_3$	30	330	1:0.32	90.8	132.8	462.1	3.48	53.4
		1000			±6.4	±8.2	±61.2	±0.10	±2.2

In the columns of Ca and P values, the first figure represents the quantities contained in the basic diet, the following figures are added Ca and P. The parentheses signify hypophosphite P, or in the Ca/P ratio, the inclusion of hypophosphite P in the total P value. The second figures in the data of results are standard deviations $S = \pm\sqrt{\frac{\Sigma(d^2)}{n-1}}$.

sensitive indicator of a deficiency or unbalance of Ca and P. Most of the control rats developed a considerable degree of baldness. This was still more accentuated in Group B, which points to a further damage caused by the change in the Ca/P ratio from 1:11 to 1:13.6. It also appeared markedly in Group K, receiving calcium gluconate without additional P, but was absent in E, where the gluconate was balanced with P. None of the other groups showed loss of hair. By comparing the Ca/P ratio in Group K (1:0.32) with those of the other groups showing baldness (Ca/P, 1:11;1:13.6) it may be concluded that the disproportion in Ca/P ratio to either extreme has a similar effect. The non-appearance of baldness in Group I may point to some assimilation of hypophosphite P, since otherwise this group would have the same Ca/P ratio as K. More data will be required to make a positive statement on this point.

Summary. Calcium hypophosphite is well suited to serve as a supplement for dietary calcium. To what extent the hypophosphite phosphorus is utilized by the organism remains uncertain. The ratio Ca/P in the diet may be varied within a wide range without affecting the calcification of the bones, provided the absolute quantities of each constituent are adequate. Extreme disproportion of Ca and P in either direction seems to cause loss of hair, as was observed in rats receiving either very low or very high relative levels of calcium.

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17073. Extra-Renal Removal of Hemoglobin from Circulation in the Rat. 1. Effect of Parenteral Bovine Albumin.

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Hemoglobin in the circulation, whether resulting from hemolysis of erythrocytes *in vivo,* or introduced by parenteral injection, is removed at least in part by excretion through the kidney. Hemoglobin filtered through the glomerulus may appear in the urine, although part is reabsorbed by the proximal tubule cells.[1-3] That hemoglobin is removed by extra-renal routes, as well, may be inferred from the accumulation of hemosiderin in reticulo-endothelial cells, when erythrocytes are destroyed at an abnormally rapid rate.

Parenteral injections of albumin might be anticipated to alter the rate at which hemoglobin is removed from the circulation by extra-renal routes. Since injected albumin is itself removed rapidly from the circulation, a nonspecific increase in protein removal might occur. On the other hand, the mechanism for protein homeostasis might be so saturated that the rate of hemoglobin removal might diminish. Overloading the reabsorption mechanism with bovine albumin is known[4] to affect the renal tubule cells so as to diminish reabsorption of hemoglobin from tubular urine.

Our interest in the extra-renal removal of hemoglobin from the circulation arose during an investigation into the mechanism of proteinuria. Using hemoglobin as an indicator,

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[2] Gerard, P., *J. Anat. and Physiol.*, 1936, **70**, 354.

[3] Rather, L. J., *J. Exp. Med.*, 1948, **87**, 163.

[4] Lippman, R. W., *Am. J. Physiol.*, 1948, **154**, 532.

we wanted to know the speed with which it left the circulation by extra-renal routes, so that the rate of urinary excretion could be better understood.

The rate of urinary hemoglobin excretion is the resultant of two opposed rates: filtration at the glomerular membrane, and reabsorption by tubular cells. Neither of these may be measured directly. However, the rate at which hemoglobin is removed from the circulation may be measured by determining total circulating hemoglobin (the product of plasma hemoglobin concentration and plasma volume) at suitable intervals. If the kidneys are surgically removed, then hemoglobin can leave the circulation only by extra-renal routes, so that the total rate of hemoglobin removal, which can be measured, becomes equal to the extra-renal rate.

Methods. In this study 48 male rats were used, each weighing about 150 g, with little variation. One group of rats received two 16 cc intraperitoneal injections of 6% bovine albumin in 0.85% sodium chloride solution at 9:00 A.M. and 4:30 P.M. on the day preceding the experiment. At 9:00 on the day of the experiment they received another such injection, and 5½ hours later they were subjected to bilateral nephrectomy under light ether anesthesia. The operative procedure was performed rapidly through 2 flank incisions. The kidney was delivered through the incision, a silk ligature was placed around the renal pedicle, and the pedicle severed. The wound was closed with Michel clips. Very little blood was lost. Previous experience has shown that rats endure this procedure with no immediate evidence of ill effects. Within a few minutes the rats are awake again, and show normal behavior.

Immediately after the completion of nephrectomy, and while still anesthetized, the rats received an intravenous injection of 141.0 mg of hemoglobin,[5] in a volume of 1.7 cc. Groups of rats were killed at intervals after injection by exsanguination from the abdominal aorta. By previous experience we know that no hemolysis occurs under the conditions of collection. Determinations were made of the serum hemoglobin concentration, total serum protein concentration, and Wintrobe hematocrit value. The serum protein concentration other than hemoglobin was calculated by difference. Another group of rats was treated in exactly the same manner, but, in place of the injections of bovine albumin, received intraperitoneal injections of 0.85% sodium chloride solution in equivalent volume, without the added protein.

Total hemoglobin concentration was measured by the method of Evelyn and Malloy.[6] Plasma volumes were calculated from the serum hemoglobin concentration and the known amount of hemoglobin injected, in the groups killed at 2 minutes. The small error introduced by the short mixing time has been neglected, as it is of smaller magnitude than other causes of error in such measurements.[7] If we assume that the circulating erythrocyte mass remains constant over short periods of time, the hematocrit value may be taken as an index of plasma volume changes at the 30 and 60 minute intervals, at which times plasma volumes were calculated from the mean initial plasma volume.

Total protein determinations were made by a modification of the Kingsley biuret method.[8]

Results. Hemoglobin was removed from the circulation with relatively great rapidity. In 60 minutes, approximately 1/3 of the quantity injected intravenously had disappeared from the circulation (Table I). The injections of bovine albumin did not appear to affect significantly the rate at which hemoglobin left the circulation.

Discussion. The speed at which physiological functions involving proteins occur is not generally appreciated. Thus, only recently, Stoerk, John, and Eisen[9] reported that the half-life of serum protein in the rat is about

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TABLE I.
Rate of Removal of Hemoglobin from the Circulation of Nephrectomized Rats.

Min. after inj.	Final serum conc. of Hb, mg/100 cc	Wintr. hematocrit	Plasma vol., cc	Total circ. Hb, mg	Hb removed mg	Rate of Hb removal mg/min./100 g BW	Serum Prot.-Hb conc., %	Total circ. Prot.-Hb, mg
			Sodium Chloride injections					
2	1492	35.3	9.5	141	0		3.54	336
30	1398	37.9	8.9	124	17	0.41 ± .19	4.53	400
60	1078	38.5	8.8	95	46	0.50 ± .06		
			Bovine albumin injections					
2	1403	33.2	10.0	141	0		6.40	640
30	1179	33.5	10.1	119	22	0.49 ± .16	6.56	650
60	1135	37.8	8.8	100	41	0.44 ± .08	7.54	664

36 hours. Control rats in this series had a total circulating protein value (other than hemoglobin) of approximately 350 mg. As the result of injections which introduced approximately 2500 mg of albumin into the circulation during a period of 30 hours (about half of the last injection remained unabsorbed at the time of autopsy), the total circulating protein rose to about 650 mg, nearly twice its previous level. Consequently a minimum of 2200 mg entered and left the circulation during the period of observation. In spite of this, no appreciable change in the rate of hemoglobin removal was detected.

Comparison of the extra-renal rate of removal with the renal excretion of hemoglobin at comparable serum concentrations indicated that, in the rats injected with sodium chloride solution, the renal excretion rate[4] was less than twice the extra-renal removal rate, while in the rats injected with bovine albumin the renal excretion rate was relatively somewhat greater. The difference in ratio depended upon the effect of bovine albumin injections, increasing the rate of renal excretion.

Summary. 1. During the hour following intravenous injection of hemoglobin in the rat, about 1/3 of the quantity injected left the circulation by extra-renal routes.

2. Intraperitoneal injection of bovine albumin, with the associated rapid passage of albumin from the circulation, did not affect the rate at which hemoglobin left the circulation.

3. The rate at which hemoglobin leaves the circulation by extra-renal routes is of comparable magnitude to, though less than, the rate of renal excretion.

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17074. Experimental Toxoplasmosis. II. Effect of Sulfadiazine and Antiserum on Congenital Toxoplasmosis in Mice.

HEINZ EICHENWALD. (Introduced by S. Z. Levine.)

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Although toxoplasmosis has been known as an infectious disease of man for over a decade, therapy of the acute condition has remained unsatisfactory. Sabin and Warren,[1,2] testing a wide variety of antimicrobial agents, reported that only the sulfonamides have any beneficial effects on the course of the disease in laboratory infected mice and rabbits. Weinman and Berne[3] extended these observa-

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[1] Sabin, A. B., and Warren, J., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 15.

[2] Sabin, A. B., and Warren, J., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 19.

[3] Weinman, D., and Berne, R., *J.A.M.A.*, 1944, **124**, 6.

tions, and by producing higher blood levels of the drugs with exceedingly large doses of sulfonamides were able to show that if used early in the course of the infection these agents are effective in curing the acute phase of the disease in adult mice. However, this method of therapy did not eradicate the infection; all treated mice remained chronic carriers, retaining virulent organisms in the brain.

In man, sulfonamide therapy for toxoplasmosis has so far given striking results in only one of the few cases in which it has been employed.[4–6]

In practice, therapy of the infection is handicapped by the fact that most recognized cases occur in newborn infants who have acquired the infection *in utero*. In consequence, widespread, irreparable, and frequently fatal damage to tissues has occurred even before birth.[7] However, we have recently observed a number of infants who seemingly had acquired the infection shortly before term, so that at the time of delivery there appeared to be little demonstrable tissue destruction. It is for these children that a successful method of treatment would be of particular value.

Experimental Procedures. The Toxoplasma strain used was the same RH strain described in an earlier paper.[8] Although it is possible to produce congenital toxoplasmosis in mice similar to that described in man, under the experimental conditions employed only about 30% of the offspring from an infected mother could be shown to harbor Toxoplasma. Of these, about three-fourths died; in the remaining infected animals the disease went on to a chronic carrier state. Because of the relatively small percentage of congenital infections produced by this method, very large numbers of animals would have been required to evaluate adequately the effect of any therapeutic agent. Therefore, in the present investigation an artificial, rather than natural, method of infecting the embryo was employed.

Adult, virgin, female mice were mated with healthy males and as soon as a vaginal plug appeared, a sign of successful copulation, were removed to individual cages. Fourteen days after copulation they were examined for signs of pregnancy. The pregnant animals were anesthetized with ether, the lower abdomen shaved, and a small incision made through the skin and peritoneum to expose the uterus. Aseptic technic was used throughout. The position of the various embryos in the uterus could easily be seen. With some practice, it was possible to insert a very fine hypodermic needle through the walls of the uterus and the fetal membranes so that it just touched the embryo. The infective material was then injected through a 0.5 cc tuberculin syringe attached to the needle. This material consisted of 0.02 cc of a 1:100 dilution of peritoneal exudate harvested from an infected, moribund, adult mouse. Each embryo was individually injected. The peritoneum was then closed with interrupted fine silk sutures, the skin with continuous silk sutures, and the wound covered with a collodion dressing.

As will be seen below, it was possible by this method to produce an infection active at birth in over 90% of the embryos injected. The pathological picture in these animals was indistinguishable from that described for those infected by transplacental transmission, except that in the latter group the degree of parasitization appeared less and the fatality rate was lower.

One hundred sixty embryos were injected on the 14th day in the manner outlined above, and the mothers allowed to give birth, which in almost every case occurred on the 18th and 19th day of gestation. Within 10 hours after birth, the offspring were separated from their mothers, divided arbitrarily into litters of 5, and placed in cages with healthy lactating foster mothers. This was done in order to eliminate the possibility of milk-borne transmission of Toxoplasma during the period of therapy since about 50% of the mothers of the injected embryos developed toxoplasmosis.[8] The litters were then divided into the following 4 groups:

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[4] Sabin, A. B., *J.A.M.A.*, 1941, **116**, 801.

[5] Zuelzer, W. W., *Arch. Path.*, 1944, **38**, 1.

[6] Robinson, P., *Ann. Paediat.*, 1947, **168**, 134.

[7] Cowen, D., Wolf, A., and Paige, B. H., *Arch. Neurol. and Psych.*, 1942, **48**, 689.

[8] Eichenwald, H., *Am. J. Dis. Child.*, 1948, **76**, 307.

TABLE I.
Treatment of Offspring from Non-Immune Mothers.

Group	Treatment begun 3rd day after birth	No. of newborn mice in group	No. of mice dead of toxoplasmosis	Avg survival period of mice dead of toxoplasmosis, days	Mice surviving experimental period (1 mo.) %	Survivors found chronic carriers, %
A	Sodium sulfadiazine 0.5 mg/g daily, 8 days	40	22	6 ± 3	45	67
B	Antiserum 0.02 cc daily, 8 days	40	28	6 ± 3	30	83
C	Sodium sulfadiazine 0.5 mg/g daily plus antiserum 0.02 cc daily, 8 days	40	5	6 ± 2	88	46
D Controls	None	40	35	4 ± 3	13	60

Group A: 40 newborn mice treated 8 days with sodium sulfadiazine, 0.5 mg/g of body weight.

Group B: 40 newborn mice treated 8 days with 0.02 cc antiserum per day.

Group C: 40 newborn mice treated with 0.5 mg/g sodium sulfadiazine per day plus 0.02 cc antiserum per day for 8 days.

Group D: 40 newborn mice receiving no treatment.

All treatment was started on the third day after birth. This day was chosen since widespread dissemination of Toxoplasma through the tissues was almost invariably present by this time.

The antiserum was obtained from mice hyperimmunized by the injection of gradually increasing doses of Toxoplasma F strain* at bi-weekly intervals, followed by increasing doses of the more virulent RH strain at weekly intervals until the mice were resistant to at least 100 minimum lethal doses of the RH strain. Two weeks after the last injection, the animals were bled, and the serum frozen at –22°C. Each day the required quantity was thawed and promptly injected intraperitoneally. Enough serum was prepared so that the entire experiment could be performed with one batch. Grace had shown previously that immune serum from rabbits also has a beneficial effect on the course of the infection in adult mice;[9] we preferred to employ mouse serum in order to avoid introducing the additional complicating factor of foreign protein reactions.

Sulfadiazine was injected subcutaneously as a 0.5% solution of the sodium salt dissolved in physiological saline solution. The daily dosage of 0.5 mg per gram of body weight of mouse was divided into 3 doses given at approximately 8 hour intervals. This dosage gives blood levels similar to those achievable safely in man, averaging about 15 mg/100 cc blood one hour after injection, 12 mg after 3 hours and 7 mg after 6 hours, as measured by the methods described by Bratton and Marshall.[10] These levels produced no apparent toxic effects in mice. The results are shown in Table I.

One month after birth, all survivors were examined for the presence of Toxoplasma by microscopic observation of Giemsa-stained material and by subinoculation of tissue suspensions into fresh mice.

Pathologically, the picture presented by the mice treated with sulfadiazine alone dif-

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* Obtained through the kindness of Dr. Isabella B. Grace of the Department of Public Health and Preventive Medicine, Cornell University Medical College.

[9] Grace, I. B., personal communication, 1947.

[10] Bratton, A. C., and Marshall, E., *J. Biol. Chem.*, 1939, **128**, 537.

TABLE II.
Course and Treatment of Offspring from Immune Mothers.

Group	Treatment begun 3rd day after birth	No. of newborn mice in group	No. of mice dead of toxoplasmosis	Avg survival period of mice dead of toxoplasmosis, days	Mice surviving experimental period (1 mo.) %	Survivors found chronic carriers, %
A	Sodium sulfadiazine 0.5 mg/g daily, 8 days	40	13	7 ± 3	68	70
B	Antiserum 0.02 cc daily, 8 days	40	22	7 ± 3	45	78
C	Sodium sulfadiazine 0.5 mg/g daily plus antiserum 0.02 cc daily, 8 days	40	4	6 ± 1	90	53
D Controls from immune mothers	None	40	26	6 ± 3	35	82
E Controls from non-immune mothers	None	40	33	4 ± 2	18	71

fered considerably from that observed in the animals treated with antiserum alone. In the latter case, active inflammatory lesions were most marked in the brain, and only relatively few scattered, apparently inactive lesions could be found in the other viscera. In the animals given sulfadiazine there were many scattered lesions throughout the liver, lung, spleen, brain, and kidney. These, however, were considerably smaller in both size and number and showed markedly less necrosis and inflammatory reaction than the widespread granulomata noted in the untreated control series. The persistence of active lesions in the central nervous system after treatment with antiserum may, perhaps, be explained best by the slight permeability of cerebral capillary walls to immune bodies, as postulated by Friedeman.[11] It was not feasible to use larger doses of antiserum.

As indicated on Table I, in the mice treated with combined antiserum and sulfadiazine, approximately half of the survivors had been cured. The remainder continued to harbor parasites, mostly in the brain, and had thus become chronic carriers.[12] The mice that died during treatment showed a widespread parasitization of tisues; in these cases the infection apparently had already been overwhelming before therapy was started.

In order to evaluate the effects of maternal antibodies on the course of toxoplasmosis in the embryo and in the newborn mouse, adult virgin female mice were injected subcutaneously with a sub-lethal dose of Toxoplasma F strain. This produced a transient disease which was followed in about 3 weeks by a strong immunity to a challenge dose of RH strain. Two weeks later the immune animals were allowed to mate with healthy male mice. On the 14th day of pregnancy, the embryos were injected *in utero* as described above. After birth, the litters were again given healthy foster mothers, and divided into 4 groups as before, plus an additional group E consisting of 40 newborn mice born from non-immune mothers. This group was given no treatment. The therapy given to the test groups was identical to that used in the previous experiment. The results are shown in Table II.

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[11] Friedeman, U., *Physiol. Rev.*, 1942, **22**, 125.

[12] Weinman, D., *J. Infect. Dis.*, 1943, **73**, 85.

The data in Table II indicate that antibodies passively acquired transplacentally by the fetus are not sufficient to protect the offspring even when sulfadiazine is given after birth, and that additional antiserum must be supplied to the progeny for optimal therapeutic effects.

Two other drugs[†] were also tried alone and in combination with antiserum but failed to show any beneficial action. These were "Promizole" (4,2′ diaminophenyl 5′ thiazole sulfone) given to newborn mice in a dosage of 0.5 mg/g per day for 8 days and "Diazone" (disodium formaldehyde sulfoxylate diaminophenyl sulfone) given in 0.1 mg/g per day for 8 days.

Discussion. A comparison of the results obtained by the various forms of treatment, as listed in Table I, readily shows that while sulfadiazine and antiserum each have a beneficial action on the course of toxoplasmosis, a combination of these two substances is considerably more effective than the use of either material alone. The reasons for this fact are probably many: one, already mentioned, may be the difficulty encountered in trying to obtain high antibody concentrations in the central nervous system; another may be the fact that humoral antibodies do not appear to have any action on intracellular Toxoplasma,[13] while extracellular organisms are not affected by sulfonamides.[1]

It should be pointed out that the histological structure of the placenta in man and in the mouse is similar if one considers the fact that in both cases only a single layer of cell separates the maternal from the fetal blood.[14] The thickness of this layer is believed to be of importance in transplacental antibody transfer.[15]

We have observed several cases of human congenital toxoplasmosis in whom, when tested a few days after delivery, the mother showed a higher neutralizing titer to Toxoplasma than did the infant. This suggests that perhaps the transfer of antibody from mother to fetus through the placental barrier is not complete, which may be one reason why the embryos from highly immune female mice are susceptible to the infection.

Even though one must be cautious not to apply unselectively data obtained from animal experimentation to human diseases, it would appear that the combined antiserum and sulfadiazine therapy should be given a clinical trial in active human congenital cases.

Summary. The effect of sulfadiazine, antiserum and a combination of these two materials on the course of congenital toxoplasmosis in mice was determined. It was shown that while sulfadiazine and antiserum individually had some beneficial action, a combination of these substances was considerably more effective than either used alone.

[†] Obtained through the kindness of Dr. Walsh McDermott of the Department of Medicine, The New York Hospital and Cornell Medical College.

[13] Sabin, A. B., and Feldman, H. A., *Science*, 1948, **108**, 660.

[14] Mason, J. H., Dalling, T., and Gordon, W. J., *Path. Bact.*, 1930, **33**, 783.

[15] Schneider, L., and Szathmary, J., *Z. Immunforsch.*, 1940, **98**, 24.

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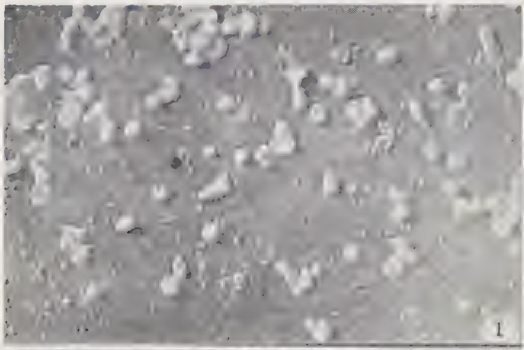
17075 P. Formation of Filamentous Forms of Newcastle Disease Virus in Hypertonic Concentration of Sodium Chloride.*

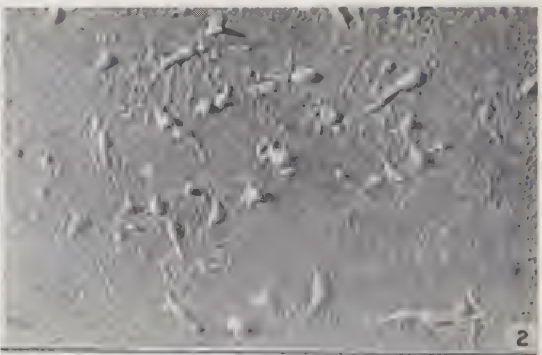
F. B. Bang. (With the technical assistance of Warren A. Hovis, Jr.)

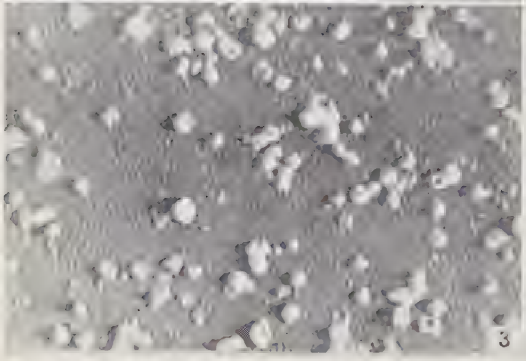
From the Department of Medicine, Johns Hopkins University School of Medicine, Baltimore.

The shape of the virus of Newcastle disease of chickens is filamentous when prepared from a saline suspension[1-3] and roughly spherical when prepared from allantoic fluid or water.[4] If the virus is partially inactivated while in water, it does not change shape when the saline is brought to .15M. This concentration of salt is sufficient to cause a change in the form of the active virus when such a suspension is dried on an electron microscope screen.[5]

Two recent chance observations added further qualifications to the system, and with subsequent experiments further indicate that the change in shape takes place in solution. A hypertonic concentration of saline is, however, needed to cause the change in shape, al-







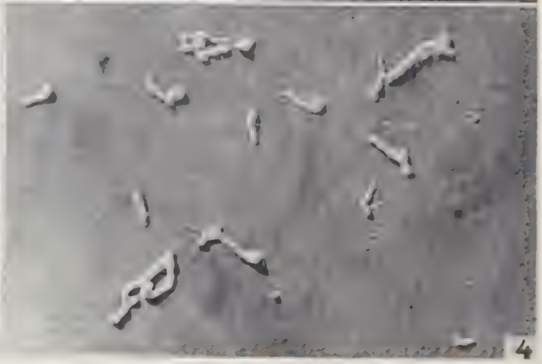


Fig. 1. Fixed in .15 M. saline by exposure to osmic acid before drying on electron microscope screen.

Fig. 2. Dried from similar suspension without fixation.

Fig. 3. Exposed to 3% saline by 2 centrifugation-washing cycles. Put back in water before preparation of screen.

Fig. 4. Same as 3, except fixed in osmic acid while in 3% saline.

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* This study was supported in part by a grant-in-aid from the National Institutes of Health, U. S. Public Health Service.

[1] Bang, F. B., Proc. Soc. Exp. Biol. and Med., 1946, **64**, 135.

[2] Cunha, R., Weil, M. L., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, T. W., *J. Immunol.*, 1947, **55**, 69.

[3] Elford, W. J., Smiles, J., Chu, C. M., and Dudgon, J. A., *Biochem. J.*, 1947, **41**, XXV.

[4] Bang, F. B., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 135.

[5] Bang, F. B., *J. Exp. Med.*, 1948, **88**, 251.

TABLE I.

Effect of Concentration of Saline on Infectivity of Suspension for Embryos and Red Cell Agglutinin.

Exp.	Conc. of saline	.8		2%		3%		4%		Untreated
		Embryo infect.	Red cell titer	Embryo infect.	Red cell titer	Embryo infect.	Red cell titer	Embryo infect.	Red cell titer	Embryo infect.
	Immediate	9.0		8.0		9.4		8.8 8.8*		8.5
1	9 days at 4°C	7.5						7.6		
	Immediate		1280		1280		640		640	
2	7 days at 4°C	7.8		8.3	2560		1280	7.4	640	

* Duplicate titration.
All titrations were carried out as in previous studies.[6]

though there is no detectable loss of activity (red cell agglutinating or embryo infectivity) of the virus.

If a 2% osmic acid solution was added to the .15M saline suspension of the virus no change in shape was detected (Fig. 1). It remained spherical. If the same .15M saline suspension of virus was dried slowly in the cold room at 4°C instead of at room temperature, again filamentous forms were not detected. This is in contrast to drying at room temperature (Fig. 2). This made it likely that a change in shape was taking place during the drying process. When we combined this idea with the assumption that osmic acid fixed the virus in the form in which it existed in solution we were led to the following experiments.

Virus freshly harvested from allantoic fluid was centrifuged at about 15,000 g for 40 minutes in an angle centrifuge. It was then resuspended in varying concentrations of saline (Table I). It was washed again by a repeated centrifugation and resuspension in the same concentration of saline. All of the preparations were then "fixed" by exposure to the osmic acid in solution. They were then transferred back to water by a third centrifugation. All of the preparations for electron microscopy were then similarly prepared by drying a drop of this final water suspension on the usual formvar screen, washing, and shadowing with chromium at angle of 4 to 1.

Figs. 3 and 4 show the change in shape which has then been detected in four separate experiments with 2 strains of the virus. We have not as yet determined the concentration of saline at which the change in shape will occur or the quantitative effect of different concentrations on different morphologies, but have shown that a marked change in shape occurs at 2% saline which is 2.5 the normal concentration of saline. This despite the lack of any evidence that the embryo infectivity was detectably reduced by placing the virus in even four per cent saline. Furthermore, the rate of loss of infectivity of the virus on remaining in the ice box was the same in different concentrations of saline.

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[6] Bang, F. B., *J. Exp. Med.*, 1948, **88**, 233.

Discussion. Since the original description of filamentous and sperm shaped virus particles[1-3] as contrasted with the spherical ones present in allantoic fluid and water we have been puzzled about the shape of the virus under physiological conditions. This is of practical interest in any attempt to study the virus in cells. Two things now seem to be fairly clear. First, the virus is probably spherical when present in solutions of physiological concentrations. This fully explains our previous failure to detect a change in shape by direct measurements of viscosity, light scattering and double refraction of flow.[5] However, it also seems clear that a change in shape will occur in hypertonic salt solutions, for elimination of the salt from the solution returns the virus to its previous shape if it has not been fixed by osmic acid.[†] To the following extent then the filamentous and sperm shaped virus particles are artifacts. They are formed by the increasing hypertonicity of the salt solution when it dries on the formvar screen. They are, however, real in that they probably exist in free solutions of somewhat hypertonic concentrations. They are at least roughly as active as the spherical forms, and do not lose activity any more rapidly than the spherical forms in water or physiological saline.

Summary. In 4 separate experiments it has been shown that the virus of Newcastle disease of chickens probably has a spherical shape when in water or .8% saline. If the concentration of saline is increased to two per cent or more the virus becomes filamentous. It may be fixed with osmic acid at this concentration and will then retain its shape when placed in water. It reverts to the spherical form when replaced in water without previous fixation. No loss of infectivity was detected during these changes in concentration of saline.

[†] Recent electron microscope observations by Elford *et al.*, *Brit. J. Exp. Path.*, 1948, **29**, 590, also indicate the presence of an osmotic membrane around the virus of Newcastle disease. We are in essential agreement with their results.

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17076. Effect of Acute Bradycardia on Pulmonary Vascular Pressures in Anesthetized Dogs.*

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From the Department of Physiology, University of Minnesota, Minneapolis.

Pulmonary edema, congestion and hemorrhage were reported in guinea pigs subjected to increased intracranial pressure; however, bilateral cervical vagotomy prior to elevation of intracranial tension resulted in a significantly lesser degree of pulmonary pathology.[1] Luisada and Sarnoff[2] employed massive, rapid venous infusion in dogs simultaneously with vagal stimulation and concluded "electrical stimulation of either the cardiac end of the cut vagi or the intact nerves favors pulmonary edema by causing extreme bradycardia".

The present authors[3] have reported bradycardia, decreased cardiac output, a significant rise in pulmonary venous pressure and pulmonary edema in dogs subjected to increased intracranial pressure. Intravenous administration of atropine after induction of the changes listed above as following maintained elevation of the intracranial pressure, reversed those changes, and significant pulmonary edema did not occur.

Methods. Mongrel dogs were anesthetized

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* This work was supported by a grant from the Life Insurance Medical Research Fund.

[1] Campbell, G. S., and Visscher, M. B., *Am. J. Physiol.*, in press.

[2] Luisada, A. A., and Sarnoff, S. J., *Am. Heart J.*, 1946, **31**, 282.

[3] Campbell, G. S., Haddy, F. J., Adams, W. L., and Visscher, M. B., *Am. J. Physiol.*, in press.

TABLE I.
Integrated Mean Pressures in Pulmonary Artery and Vein in Relation to Heart Rate Changes.

	Wt. of dog, kg		Before vagal stimulation beats/min.	mm/Hg	During first min. of vagal stimulation beats/min.	mm/Hg	After vagal stimulation beats/min.	mm/Hg
1	15.7	Heart rate	216		72		240	
		Pulmonary venous pressure		4.0		14.8		4.5
		Heart rate	180		60		200	
		Pulmonary artery pressure		12.8		9.3		12.8
2	18.6	Heart rate	144		72		144	
		Pulmonary venous pressure		5.0		9.5		5.3
		Heart rate	144		48		130	
		Pulmonary artery pressure		16.0		14.0		17.3
3	24.2	Heart rate	180		72		180	
		Pulmonary venous pressure		6.3		11.3		6.3
		Heart rate	180		72		180	
		Pulmonary artery pressure		20.0		18.0		19.8
4	20.0	Heart rate	168		60		168	
		Pulmonary venous pressure		9.0		13.8		5.0
		Heart rate	168		60		180	
		Pulmonary artery pressure		16.3		15.3		17.5
Average		Heart rate	178		69		183	
		Pulmonary venous pressure		6.1		12.4		5.3
		Heart rate	168		60		173	
		Pulmonary artery pressure		16.3		14.2		16.9

with sodium pentobarbital, 30 mg/kg and No. 10 radio-opaque whistle-tipped ureteral catheters were put into the jugular vein and carotid artery. The catheters were passed into the pulmonary artery and pulmonary vein respectively with fluoroscopic aid. A small cannula was placed in the thoracic cavity and intrathoracic, pulmonary artery and pulmonary vein pressures were recorded with standard resistance wire pressure transmitters (Statham strain gauges).† A more detailed description of the technic employed was reported elsewhere.[4]

Femoral arterial pressure was measured with a mercury manometer, and jugular venous pressure was measured with a manometer filled with 5% sodium citrate. Heart rates were counted from the pulmonary artery pressure records. Integrated mean pulmonary vascular and intrathoracic pressures were measured with a compensating polar planimeter, and all pulmonary pressures reported herein are expressed in relation to intrathoracic pressure as zero. All dogs had an indwelling tracheal cannula.

A Harvard inductorium was used for faradic stimulation of the cardiac ends of the cut vagus nerves and acetylcholine and/or acetyl-beta-methylcholine were also employed in order to produce bradycardia.

Results and discussion. Faradic stimulation of the cardiac ends of the cut cervical vagi was employed to produce cardiac slowing to one-half or one-third of the control rate. There was a prompt elevation of mean pulmonary venous pressure, and a smaller fall in mean pulmonary artery pressure. Details of these observations are presented in Table I. The average values for the 4 dogs studied show a 6.3 mm Hg rise in integrated mean pulmonary venous pressure concomitant with the slowing of the heart rate from 178/minute to 69/minute. Simultaneously with the acute bradycardia in the same dogs the pulmonary artery pressure was lowered 2.1 mm Hg. It may be noted that in Dog No. 1 the integrated mean pressure recorded was higher in the pulmonary vein than in the pulmonary artery. Several factors complicate this measurement, for example, the catheter aperture opens into the stream of blood in the vein and is oppositely situated in the artery. As a result the kinetic energy factor is added to lateral pressure in the first case and subtracted in the second. For this reason one cannot, without velocity measurements or their equivalent, give absolute lateral pressure values. It is possible, however, that with bradycardia the pulmonary vascular pressure gradient direction is actually reversed in diastole. A slow diastolic rise in pulmonary artery pressure seen only in this case would conform with this interpretation. The maximum systolic pulmonary arterial pressure is greatly increased in bradycardia.

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† Statham Laboratories, 9328 Santa Monica Blvd., Beverly Hills, Calif.

[4] Haddy, F. J., Campbell, G. S., Adams, W. L., and Visscher, M. B., *Am. J. Physiol.*, in press.

In one case the cardiac output was measured by the direct Fick method before, during and immediately after vagal stimulation. The values obtained for the cardiac outputs were 3.2, 2.0 and 2.4 L. per min. in the 3 situations in the order named. The systemic venous pressures rose 2-6 cm H_2O during the vagal stimulation periods and the mean femoral arterial pressure fell slightly although the pulse pressure increased.

In two experiments with acetylcholine and one with acetyl-beta-methylcholine administered in such doses as to lower the heart rate to a half or less of the control value similar changes in pulmonary vascular pressures were recorded.

The observations recorded herein are entirely in harmony with predictions from classical hemodynamics. They are being reported primarily because they have a bearing on the mechanism of production of pulmonary congestion and edema, in states of bradycardia. In one respect the effects of acute vagal stimulation appear to differ from the results when comparable bradycardia occurs gradually after increasing the intracranial pressure. In the latter case the pulmonary artery pressure rises, in contrast to the fall reported herein for acute vagal bradycardia. It should be noted that when vagal stimulation was continued for 10 minutes, the bradycardia being maintained, the pulmonary artery pressure rose to values above the control level. The pulmonary venous pressure remains elevated. The mechanism of the delayed rise in pulmonary artery pressure has not been analyzed. It is obvious from the observations reported here that simple bradycardia can account for the elevation in pulmonary venous pressure seen when cardiac slowing results from elevations in intracranial pressure. Since in the latter situation the ensuing pulmonary edema is correlated with the pulmonary venous pressure[3] one can also link the genesis of the edema with bradycardia.

Summary. The bradycardia produced by vagal stimulation and by the administration of acetylcholine or acetyl-beta-methylcholine resulted in an elevation of mean pulmonary venous pressure and a small fall in mean pulmonary artery pressure. These observations are discussed in relation to the genesis of pulmonary edema in situations in which bradycardia occurs.

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17077. Production of Anemia in Swine Fed Purified Diets and Sulfasuxidine.*

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From the Department of Medicine, College of Medicine, University of Utah, Salt Lake City.

In 1946 it was reported from this laboratory[1] that a pig maintained on a diet in which purified casein (Borden's Labco "vitamin-free") was substituted for crude casein (Sheffield's New Process) and to which 2 per cent

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* Aided by a grant from the United States Public Health Service and the Upjohn Company, Kalamazoo, Mich.

[1] Cartwright, G. E., Wintrobe, M. M., and Humphreys, S., *J. Lab. and Clin. Med.*, 1946, **31**, 423.

sulfasuxidine was added, failed to grow normally and developed a normocytic anemia. Following treatment with a highly purified antipernicious anemia liver extract, growth was resumed and, after a definite although not marked reticulocytosis, the blood returned to normal. This observation has been confirmed by Cunha *et al.*[2] However, Welch *et al.*[3] fed a similar diet to two 63 day old pigs and failed to observe either retardation of growth or anemia. Cunha *et al.* reported that the development of the anemia was prevented by the administration of pteroylglutamic acid and, to a lesser extent, by antipernicious anemia liver extract.

More recently it has been demonstrated in this laboratory[4,5] as well as by Welch and Heinle,[6–8] that swine fed a similar purified casein diet supplemented with sulfasuxidine to which a folic acid antagonist is added, develop severe macrocytic anemia, leukopenia, slight thrombocytopenia and hyperplasia of the bone marrow. In the bone marrow there is an increase in immature nucleated red cells which, furthermore, resemble the megaloblasts seen in the marrow of patients with pernicious anemia. This anemia responds rapidly to the administration of pteroylglutamic acid. Purified liver extracts manifest some hemopoietic activity in such animals but the activity is considerably less than that of pteroylglutamic acid.[4,5]

The purpose of the present study was to determine if our original observation could be confirmed and, if so, whether the anemia so produced differs either morphologically or in its response to therapy, from the anemia produced in swine given a folic acid antagonist.

Experimental. A total of 75 weanling Chester-White pigs, 21 to 28 days of age were used. The animals were divided into three groups as follows:

Group	No. pigs	Type casein	Sulfasuxidine‡ %
I	12	Purified*	2
II	42	Crude†	0
III	21	”	2

All animals received the following vitamins: A, D, thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, choline and inositol. In addition, those in group II were given pteroylglutamic acid, animals in group I were given para-aminobenzoic acid and those in groups II and III were given biotin. The percentage of casein fed is indicated in Tables I and III. Full details of the experimental methods, the composition of the basal diet and the amounts of vitamins administered have been given in previous reports.[1,4,5,9]

Results. The hematologic studies in the 12 animals in group I are presented in Table I. Anemia developed in each instance. The anemia was mild (volume of packed red cells 30 to 35 ml/100 ml) in 5 pigs, moderately severe (volume of packed red cells 25 to 30) in 4 pigs, and severe (volume of packed red cells less than 25) in 3 pigs. The anemia was normochromic and in general normocytic although the mean corpuscular volume was greater than 65 $c\mu$ in 4 pigs. Differential cell counts on marrow aspirated from the sternum revealed a decrease in the leukocyte-erythroid ratio with many immature nucleated red cells present. These cells did not differ significantly in their morphologic characteristics from those observed in the marrow of

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[2] Cunha, T. J., Colby, R. W., Bustad, L. K., and Bone, J. F., *J. Nutrition*, 1948, **36**, 215.

[3] Welch, A. D., Heinle, R. W., Sharpe, G., George, W. L., and Epstein, M., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 364.

[4] Cartwright, G. E., Fay, J., Tatting, B., and Wintrobe, M. M., *J. Lab. and Clin. Med.*, 1948, **33**, 397.

[5] Cartwright, G. E., Tatting, B., Ashenbrucker, H., and Wintrobe, M. M., *Blood*, 1949, **4**, 301.

[6] Heinle, R. W., Welch, A. D., George, W. L., Epstein, M., and Pritchard, J. A., *J. Lab. and Clin. Med.*, 1947, **32**, 1398.

[7] Welch, A. D., Heinle, R. W., Pritchard, J. A., and Solis, H., *Fed. Proc.*, 1948, **7**, 300.

[8] Heinle, R. W., Welch, A. D., and Pritchard, J. A., *J. Lab. and Clin. Med.*, 1948, **33**, 1647.

[*] Either Borden's Labco "vitamin-free" casein or Sheffield's special alcohol extracted casein.[1]

[†] Sheffield's New Process (Crude) Casein.

[‡] Kindly furnished by Sharp and Dohme, Inc., Philadelphia, Pa., through the courtesy of Dr. W. A. Feirer.

[9] Wintrobe, M. M., Miller, J. L., and Lisco, H., *Bull. Johns Hopkins Hosp.*, 1940, **67**, 377.

TABLE I.
Hematologic Observations in Pigs Receiving Purified Casein and Sulfasuxidine (Group I).

Pig No.	Casein in diet, %	Day of exper.	RBC millions per c.mm.	Hgb. gm, %	VPRC ml/100 ml	MCV, cμ	MCH, γγ	MCHC, %
9–31	18	135	3.70	6.6	21.0	57	18	31
32	18	206	3.93	9.4	28.0	71	24	34
52	26	136	5.19	10.4	30.5	59	19	33
53	26	85	5.64	9.0	30.0	54	16	33
66	12	130	3.57	8.1	24.5	69	23	33
67	12	130	4.67	10.1	30.0	64	22	34
69	18	130	3.03	6.9	19.5	64	23	35
10–66	26	190	4.68	12.4	34.8	74	26	36
68	26	190	4.15	11.3	32.0	77	27	35
69	26	139	4.52	9.1	28.2	62	20	32
71	26	153	4.60	9.2	28.0	63	21	33
82	26	94	5.13	9.9	29.6	58	19	33

VPRC refers to volume of packed red cells; MCV refers to mean corpuscular volume; MCH refers to mean corpuscular hemoglobin; MCHC refers to mean corpuscular hemoglobin concentration.

TABLE II.
Response of the Anemia in Group I to Pteroylglutamic Acid (PGA) and Liver Extract Therapy.

			Before therapy		After therapy			
					Retic peak		Vol. pkd. RBC	
Pig No.	Substance	Dose I.M.	VPRC, ml/100 ml	Retics, %	%	day	ml/100 ml	day
9-31	PGA	50 mg	24.5	0.6	5.4	3	37.0	17
32	PGA	50 mg	28.0	0.8	3.6	4	42.5	10
66	PGA	50 mg	24.5	1.0	16.0	5	44.0	14
67	PGA	50 mg	30.0	1.0	11.8	3	40.0	18
69	PGA	50 mg	19.5	0.2	8.0	7	39.5	14
10-66	PGA	20 mg	35.0	2.0	6.0	7	45.5	14
68	Liver extr.*	150 u	32.0	0.8	5.0	2	48.0	28
69	” ”	150 u	28.2	2.0	8.0	5	35.0	14
69	PGA	20 mg	31.0	0.8	12.0	5	45.5	21
71	Liver extr.*	75 u	27.0	2.2	6.0	5	37.0	14
71	PGA	20 mg	35.0	1.0	14.0	5	42.0	11

* Parke, Davis and Company, 15 U.S.P. units per ml.
u refers to U.S.P. units of liver extract.

pigs receiving a crude methyl folic acid antagonist.[4,5] However, in general the changes were not as marked.

The results of therapy with pteroylglutamic acid and with liver extract are presented in Table II. The administration of pteroylglutamic acid resulted in a significant reticulocytosis and a rise in volume of packed red cells to normal. The administration of liver extract was followed by a slight reticulocytosis and a significant although suboptimal rise in volume of packed red cells. In two of the pigs so treated the administration of pteroylglutamic acid resulted in a second reticulocytosis and a further increase in the volume of packed red cells.

The hematologic studies in the animals in groups II and III are presented in Table III where are compared the volumes of packed red cells and the mean corpuscular volumes of pigs fed various proportions of crude casein with and without pteroylglutamic acid and sulfasuxidine. It is clear from these data that, like the animals receiving purified casein (group I, Table I), the inclusion of sulfasuxidine in a pteroylglutamic acid-“free” crude casein diet also resulted in the development of significant anemia (group III). The anemia was comparatively more severe in the pigs fed casein at a 10 per cent level than in those receiving a diet containing 26 per cent casein. In animals given a folic

TABLE III.
Hematologic Observations in Pigs Receiving Crude Casein.
(Groups II and III). Mathematical Averages and Ranges.

Dietary casein, %	Group II			Group III		
	No. pigs	VPRC, ml/100 ml	MCV, c.μ.	No. pigs	VPRC, ml/100 ml	MCV, c.μ.
26	10	42.0 (39-47)	53 (51-63)	5	37.3 (31-41)	58 (57-63)
18	10	37.1 (34-41)	56 (50-62)	10	29.7 (25-37)	58 (51-62)
10	22	35.7 (34-40)	55 (49-65)	6	24.6 (21-32)	56 (51-63)

Group II received pteroylglutamic acid and no sulfasuxidine.
Group III received no pteroylglutamic acid but were given sulfasuxidine.
VPRC refers to volume of packed red cells.
MCV refers to mean corpuscular volume.
Figures in parentheses represent range.

acid antagonist, it has also been found that pteroylglutamic acid deficiency develops more rapidly on a diet low in protein.[5]

There were certain dissimilarities between the anemia produced on a crude casein diet and the anemia produced on the diet containing purified casein. No anemia (volume of packed red cells greater than 35 ml/100 ml) was present in 6 (29 per cent) of the pigs fed a crude casein diet. The anemia when it developed was mild (volume of packed red cells, 30 to 35) in 4 pigs (23 per cent), moderately severe (volume of packed red cells, 25 to 30) in 8 (38 per cent), and severe (volume of packed red cells less than 25) in only 2 (10 per cent). Thus the anemia was not produced as consistently, nor was it as severe, as in pigs fed the purified casein. It developed somewhat more slowly and when present, was normocytic. Furthermore, after the development of anemia in the animals fed crude casein in each instance reticulocytosis appeared spontaneously and this was followed by the complete disappearance of the anemia (Fig. 1). The reticulocytosis was gradual in its development and persisted for several weeks, giving a low, flat curve. A sharp peak, such as follows the administration of pteroylglutamic acid (Fig. 1), was not observed. The degree of reticulocytosis varied from 6 to 23 percent and usually reached 10 to 15 per cent.

Summary. Pigs maintained on a diet in which purified casein was substituted for crude casein and to which 2 per cent sulfasuxidine was added developed anemia which responded partially to purified liver extract and completely to pteroylglutamic acid. This anemia resembles that which has been produced in pigs given a folic acid antagonist.

Pigs maintained on a diet containing crude casein and to which 2 per cent sulfasuxidine was added developed less pronounced anemia which disappeared spontaneously.

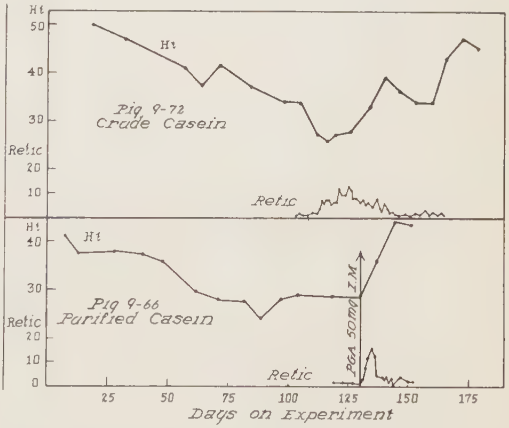

FIG. 1.

Pig 9-72 (Group III) received crude casein (12%). Note the development of anemia and the spontaneous reticulocytosis with relief of the anemia. Pig 9-66 (Group I) received purified casein (12%). Note the persistent anemia and the rapid response with a sharp reticulocyte peak which followed the administration of pteroylglutamic acid.

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17078. Recording of Intracavity Potentials Through a Single Lumen, Saline Filled Cardiac Catheter.

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The purpose of this report is to present a simple new technic whereby the electrical potential within the human or experimental animal heart can be accurately recorded using a single lumen catheter instead of the usual indwelling wire conductor type of catheter. The column of blood or saline in the catheter acts as the linear conductor to transmit electrical currents arising in the heart.

Apparatus and methods. The technic requires a simple electrode attachment consisting of a 2-4 cm length of silver or German silver wire, an adapter to fit into the arm end of the catheter, and an ordinary battery clip. Two models are illustrated (Fig. 1) Both produced identical records. Since a secure junction is essential for tracings free from artefacts, the model with the metal adapter is recommended for general use and especially whenever high intracatheter pressures exist as in retrograde arterial catheterization of the left side of the heart, or in right heart catheterization of patients with pulmonary hypertension.

The catheter is passed in the usual fashion into the cavity of the heart and is kept patent with a slow drip of heparinized physiological saline or glucose. To obtain intracavity potentials, the above described electrode attachment is connected to the open arm end of the catheter. The exploring terminal of the electrocardiograph is connected to the battery clip of this electrode, and Wilson's central terminal serves as the indifferent electrode. The standardization has been so adjusted that 1 millivolt causes a deflection of 0.8 to 1.2 cm. A radio amplifier type of electrocardiograph is preferred. The electrocardiographic tracing thus obtained represents the potential changes occurring only at the tip of the catheter.

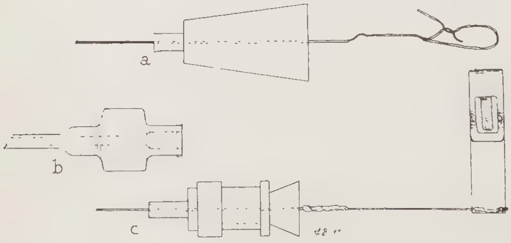

Fig. 1.

(a) Rubber stopper modified to insert into arm end of catheter (b). (c) Is metal syringe adapter with rubber center core. Through each adapter a German silver wire is passed to project 2-4 cm. into the saline filled lumen of the arm end of the catheter. Exploring terminal of ECG is connected to battery clip.

There is a certain amount of admixture of blood with the saline in the tip of the catheter. We have allowed the catheter to fill with blood and compared these tracings with those obtained with a saline flushed catheter. Heparinized saline serves as a more satisfactory conducting medium because of the lesser tendency for blood to clot within the catheter. It is our impression that *clotted* blood has a greater resistivity, as reflected by a diminution in amplitude of the deflections recorded following only minor clot formation. A study of the comparative electrical conductivity of serum, and liquid and clotted blood is planned in the immediate future.

Results. Intracavity potentials have been satisfactorily recorded in 14 patients by this technic. To prove that actual intracavity potential was being recorded, a double lumen electrode catheter was passed into the right ventricle. One lumen contained a wire which extended to the tip, and constitutes the usual cardiac electrode. The electrocardiogram obtained from this cardiac wire electrode was compared with the record simultaneously obtained from the column of blood or saline in the second lumen. In every instance intracavitary potentials were obtained. In general the form of the complexes recorded from each was identical (Fig. 2). Occasionally minor

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* Fellow of the National Institute of Health.

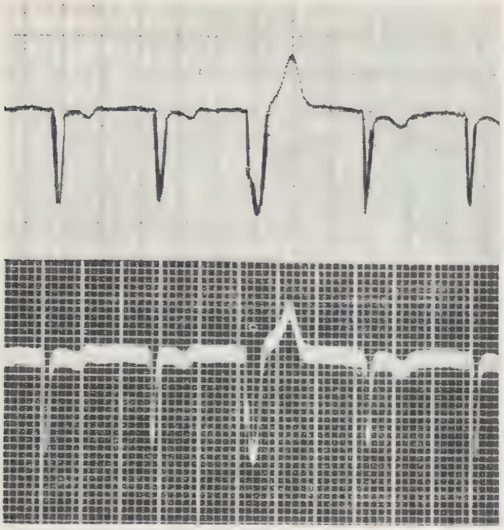

FIG. 2.
Intracavitary potentials recorded simultaneously with a double lumen catheter in right ventricle. Upper tracing was recorded with saline as conductor, lower with usual wire electrode. Form of normal and premature beats are identical. Retouched for publication.

FIG. 3.
Injury in man produced by pressure of tip of double lumen catheter against the endocardium of the right ventricle. As the catheter is withdrawn injury currents disappear. The upper curves were obtained with the saline in one lumen as a conductor. Lower curves were recorded from an indwelling wire which extended to the tip of the second lumen. Note greater ST elevation in the upper record.

differences in contour occurred, due to slight differences in the spatial orientation of the wire electrode tip and the opening of the catheter within the heart. For example, in one patient (Fig. 3) the wire electrode tracing showed definite S-T elevation whereas the open catheter tracing showed more marked S-T elevation, indicating greater currents of injury at its tip, presumably produced by the open end of the catheter resting squarely against the endocardium.[1] Identical records were obtained when the catheter was withdrawn less than 1 cm. Similarly, simultaneous tracings were also recorded at different levels in the right ventricle, right atrium, and superior vena cava.

Although contour was similar, there was a difference in the amplitude of the complexes when the same standardization was used. Consistently, the tracings were larger when recorded through the usual wire electrode, due to the greater resistance of saline or blood.[2] Since the calculated specific resistance of physiological saline at 25°C is approximately 65 ohm cm[3] and that of copper wire 1.7×10^{-6} ohm cm, this difference in amplitude is quite understandable.

There are certain precautions to be observed if satisfactory intracavity tracings are to be recorded from the single lumen catheter without use of a wire. Alternating current interference is frequently encountered and means should be taken to eliminate it. Small a-c voltage is easily picked up from nearby alternating current circuits or machines. A loose connection or a high resistance at the junction at the arm end of the catheter between the saline and the adapter wire favors a-c disturbance. Extreme care must also be taken to keep the operative field dry. If the arm end of the catheter is in contact with a damp field, the electrical potentials recorded will be a summation of that arm's potential and the intracavitary potential subtended by the tip of the catheter. Thus, we have purposely converted a typical intraventricular electrocardiogram to a left arm unipolar tracing by simply placing the arm end of the catheter electrode connection in contact with a saline-saturated towel on the patient's left arm.

There are certain advantages in the use of this technic. A single lumen catheter can thus be used to obtain blood samples, pressure curves, and intracavitary potentials. Some adults and most children have veins too small to allow passage of the usual double lumen catheter electrode.

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[1] Hellerstein, H. K., and Katz, L. N., *Am. Heart J.*, 1948, **36**, 184.

[2] Kaufman, W., and Johnston, F. D., *Am. Heart J.*, 1943, **26**, 42.

[3] MacDougall, F. H., Physical Chemistry, New York, Macmillan Co., 1947, pp. 468, 475.

During the procedure of introducing the catheter, electrocardiographic tracings from the tip of the catheter are valuable in determining the exact anatomical location of the tip. We have found that changes in the contour of the P and QRS complexes may be a more reliable index of the location of the tip of the catheter than either the fluoroscope or the pressure curve, especially in the region of the lower right atrium near the tricuspid valve, or in the coronary sinus.

Summary. A new technic is described to obtain intracavitary cardiac potentials through the single lumen saline filled cardiac catheter without use of the usual copper wire electrode. If the exploring terminal of an electrocardiograph is connected to the open arm end of a column of blood or saline in a catheter which has been introduced into the cavity of the heart, and if the indifferent electrode is connected to a central terminal, the completed curve will represent chiefly the fluctuations of intra-cardiac potential at the tip of the catheter.

Prior to submitting this article for publication we were unaware of the report of Kisch *et al.*, *J. Mt. Sinai Hosp.*, 1948, **15**, 257, who described a similar technic although he advocates the introduction of a thin wire into the catheter to avoid a.c. interference.

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17079. Reflex Modulation of Heart Rate on Closure and Opening of an A-V Fistula.[*][†]

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Branham[1] described the well known slowing of the heart rate which ensues upon temporary obliteration of an A-V fistula by digital compression. The reverse phenomenon, *viz.*, increase in heart rate, also takes place when the pressure is released. That the vagus nerves are the efferent pathway of the reflex arc responsible for this reaction has been demonstrated by Weber,[2] Lewis and Drury,[3,4] and Holman,[5] by use of atropinization techniques. While it is logical to infer that the sinus and aortic nerves constitute the afferent arcs from the arterial side, the possibility that afferents from the venous side may also be concerned has not been excluded. Since closure of an A-V fistula causes a fall, and opening a rise of central venous pressure,[6] such changes in pressure could evoke reflex alterations in heart rate if the Bainbridge reflex is operative. The question as to which afferent pathways are dominantly and subdominantly concerned was therefore resubmitted to experimental study.

Method. Dogs varying in weight from 10 to 12 kilos were anesthetized with chloralose because this anesthetic increases the sensitivity of the cardio-inhibitory mechanism, whereby the vagal tone is well maintained. An

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[*] Supported by a grant from the Life Insurance Medical Research Fund.

[†] Condensed report of thesis submitted in partial fulfillment of requirements for an M.S. degree, Western Reserve University Medical School, Cleveland, Ohio.

[‡] Indian Government Fellow in Physiology.

[1] Branham, H. H., *Internat. J. Surg.*, 1890, **3**, 250.

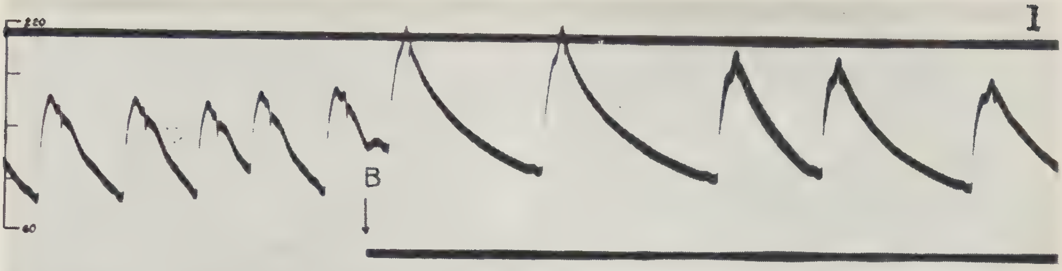
[2] Weber, A., *Munchen med. Wchnschr.*, 1917, **64**, 409.

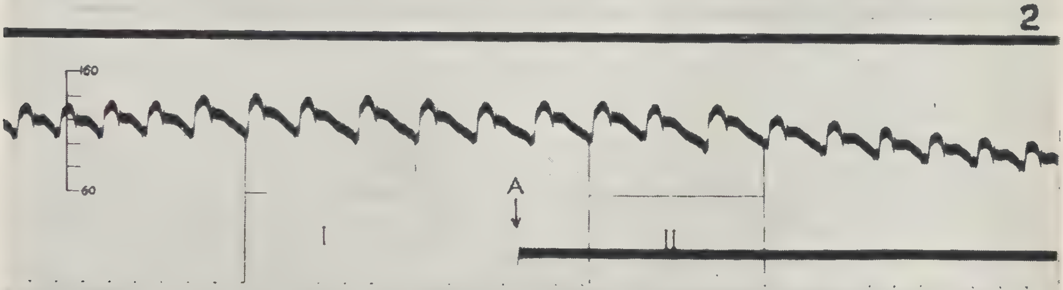
[3] Lewis, T., and Drury, A. N., *Heart*, 1923, **10**, 301.

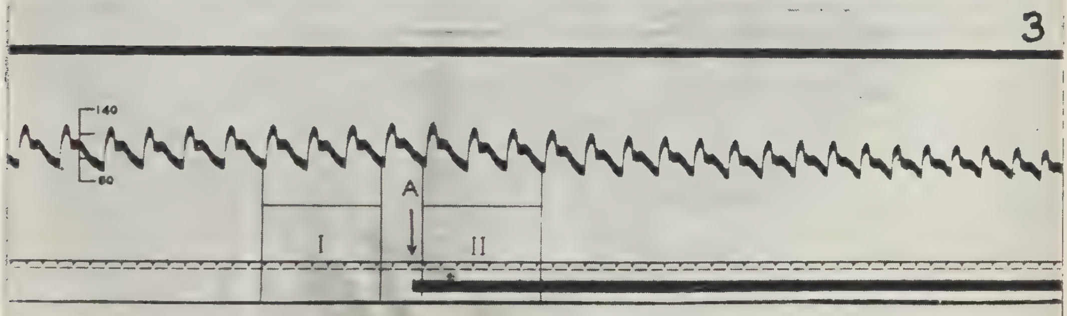
[4] Lewis, T., and Drury, A. N., *Heart*, 1923, **10**. 365.

[5] Holman, E., *Ann. Surg.*, 1924, **80**, 801.

[6] Heringman, E. C., Davis, H. A., and Rives, J. D., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 371.







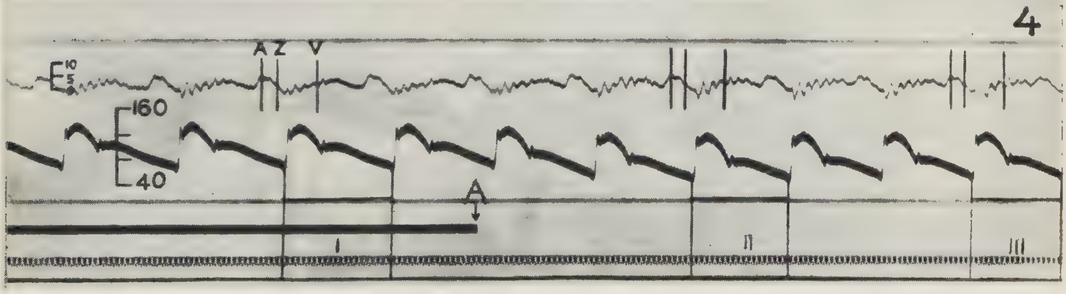


FIG. 1. Aortic pressure pulses showing the effect of closing (B) an A-V shunt on aortic pressure and heart rate. Time, 0.2 sec.

FIG. 2 and 3. Effect of closing an A-V shunt (A) during stabilization of arterial pressure by simultaneous withdrawal of blood. Time, 0.2 sec.

FIG. 4. Effect of opening an A-V shunt (A) on right atrial pressure (upper) and aortic pressure (lower). Time, 0.02 sec.

initial dose of 75 mg per kilo was followed by 15 mg per kilo every hour. Bilateral anastomoses were made between the femoral artery and femoral vein of each side. These vessels were first cannulated and then joined by rubber tubing. A hemostat was used to open and close the circuit thus produced. Optical manometers of adequate sensitivity and calibrated against a mercury manometer under static conditions were used to register aortic pressure pulses. Mean blood pressure was determined by planimetry of 3 successive heart cycles.

Experiments. Control reactions were first recorded by simply opening and the closing arterio-venous shunts. It was observed that there was always an increase in heart rate with fall of arterial pressure on opening the shunts, and a lowering of the heart rate with rise of arterial pressure on closing the shunts. Fig. I illustrates an experiment in which closing of the shunts at B caused diastolic pressure to rise immediately and the heart rate to slow to a pronounced degree.

In all of these control experiments it was found that the change in heart rate on opening of the shunts took place within 1 to 5 beats (average 1.4 seconds), and maximum slowing developed within 3 beats after closure of the shunts. The fact that this slowing of the heart rate is always preceded by a rise of pressure on closing the shunts naturally suggests that the carotid sinus and aortic nerves constitute the afferent pathways.

To test this hypothesis an effort was made to maintain arterial pressure constant after closure of the shunts. By repeated trials it was found that this could be accomplished in a reasonably satisfactory number of experiments by inserting a Y-tube into one of the A-V anastomoses, through which blood could be withdrawn from the animal at the moment that the venous limb of the Y was closed. By this expedient the animal could be bled from the femoral artery at a rate adjusted to counteract the normal rise in arterial pressure. While it was difficult to maintain an absolutely constant level of mean arterial pressure before and after closure of the shunts in all cases, a sufficient number of records were realized in which no changes or only insignificant alterations in arterial pressure took place. A plot of all experiments in which arterial pressure rose, essentially remained unchanged, or fell, indicated that slowing of the heart rate always accompanied a definite rise in arterial pressure. The change in heart rate on fall of arterial pressure was not so uniform. When this was less than 5 mm Hg acceleration was not a constant feature. In 4 cases moderate acceleration was observed, and in 6 a definite slowing. Two records free from sinus arrhythmia are reproduced in Fig. 2 and 3. In both of these the change in heart rate is strikingly small as compared to that shown in Fig. 1. Calculation showed that in the curve of Fig. 2 a slight slowing of the heart rate takes place without any rise in arterial pressure. Actual measurement revealed a reduction during period II which would be equivalent to 5 beats per minute as compared to period I, in spite of a fall in mean pressure of 3 mm Hg. In Fig. 3 the slowing is even less, being equivalent to two beats per minute with a fall of mean arterial pressure of only 1 mm Hg.

It is tempting to accept the corollary that the small changes in heart rate which still persist when arterial pressure remains stabilized is occasioned by the Bainbridge reflex. Before this assumption may be accepted, however, it is important to establish that venous pressure is in fact reduced by closure of a shunt. For this reason, several experiments were carried out in "open chest" animals in which right atrial pressure was recorded by an optical manometer. No convincing evidence of a significant change of right atrial pressure was obtained. Fig. 4 shows one of the curves obtained on opening the A-V shunt. It is obvious that right atrial pressure is not significantly affected. Careful measurements of the original curves revealed a rise of pressure equal to 6 mm H_2O at A, of 8 mm H_2O at Z, and no alteration at V.

Discussion. In a sense it was disappointing to find that the A-V shunts employed in these experiments were apparently not large enough to cause a sufficient change of right atrial pressure to test the operation of the Bainbridge reflex. They do not therefore exclude the possibility that such reflexes may operate when larger shunts exist which apparently do cause an elevation of right atrial pressure.[7] This occurrence was fortunate in another way; it pointed out the difficulty of establishing the existence of such a reflex in a crucial way. Had a material fall of right atrial pressure taken place in these experiments while arterial pressure was kept from rising, no one would have contested the conclusion that the Bainbridge reflex is responsible for the degree of slowing shown in Fig. 2 and 3. Having unwittingly devised an experiment in which neither right atrial nor arterial pressure altered significantly, it becomes apparent that these changes in heart rate must be attributed to still a different factor.

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[7] Holman, E., and Kolls, A. C., *Arch. Surg.*, 1924, **9**, 837.

The possibility has been suggested[8,9] that differences in pulse pressure, aside from alterations in mean arterial pressure, may operate to cause reflex alteration in heart rate. However, this factor is also excluded in the curves of Fig. 2 and 3, in which the pulse pressure likewise remains unaltered before the heart rate slows. In short, the slight subsidiary changes in heart rate which are still found upon compression of an A-V fistula remain undetermined.

Summary. An attempt was made to determine whether reflexes other than those from arterial pressor receptors are concerned in modulation of the heart rate when an A-V fistula is closed or opened. In dogs under chloralose anesthesia, bilateral A-V shunts were established between femoral arteries and veins, one of which being so arranged that blood could be drained from the animal from the arterial side during closure of the shunt in an amount sufficient to stabilize arterial pressure at a fairly constant level.

In such experiments it was found that slight slowing of the heart rate still occurs on closing a shunt even when arterial pressure falls a little and the pulse pressure is unaffected. Since supplementary experiments of a similar type failed to reveal significant changes in right atrial pressure, changes in central venous pressure were also excluded. These experiments call attention to the difficulty of establishing the operation of the Bainbridge reflex in a crucial manner.

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[8] Bronk, D. W., *Harvey Lec.*, 1934, **29**, 245.

[9] McCrea, F. D., and Wiggers, C. J., *Am. J. Physiol.*, 1933, **103**, 417.

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17080. A Newcastle Disease Virus (NDV) Hemolysin.

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From the Department of Epidemiology, Harvard School of Public Health.

The first observations of a virus hemolysin were those by Morgan, Enders, and Wagley[1] in regard to mumps virus. An hemolysin associated with Newcastle Disease Virus (NDV) has not hitherto been described, although its presence has been observed.[2] The methods employed in the present investigation were modifications of those described by Morgan *et al.*[1]

Dr. Erwin Jungherr kindly supplied the California strain of NDV (no. 11,914) used in all experiments. Infected allantoic and amniotic (AA) fluids were harvested together 36 hours after intra-allantoic inoculation of 10-day-old embryonated eggs with NDV. A single pool of fluids, stored in CO_2 ice, was used throughout. In hemagglutination (HA) tests, 0.5 cc of 0.25% hen erythrocytes was added to an equal amount of each virus dilution. Readings were taken after 1¼ hours at room temperature. Two per cent cells were used in hemolysis (HL) tests which were incubated for 2 hours at 37°C. Except for 1 test run at pH 6 (Table I), all dilutions were made in isotonic phosphate buffer pH 7.2.

Some properties of the NDV hemolysin are illustrated in Table I. Hemagglutination and hemolysis tests run on the original AA fluids served as controls. These were of especial

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[1] Morgan, H. R., Enders, J. F., and Wagley, P. F., *J. Exp. Med.*, 1948, **88**, 503.

[2] Florman, A. L., personal communication.

TABLE I. Effects of Heat, pH, and Species Differences in Erythrocytes on Hemagglutination and Hemolysis by Newcastle Disease Virus (NDV).

		Test	Dilutions of NDV											Buffer cont.
			8	16	32	64	128	256	512	1024	2048	4096	8192	
Control	pH 7.2	HA	4*	4	4	4	4	4	4	4	4	3	0	0
Titration		HL	4	4	4	3	2	1+	1	±	0	0	0	0
Effect of heat														
51°C 15 min.		HA	4	4	4	4	4	4	4	4	3	0		0
		HL	3	3	3	2+	2	1+	1	±	0	0		0
60°C 8 min.		HA	0	0	0	0	0	0	0	0	0	0		0
		HL	0	0	0	0	0	0	0	0	0	0		0
Effect of pH														
pH 6		HA	4	4	4	4	4	4	4	4	4	0		0
		HL (Brown)	4	3	2	2—	1+	1	±	0	0	0		0
Erythrocytes different species														
Sheep		HA	4	4	4	4	4	4	4	0	0	0		0
		HL	4	4	3	2	1	0	0	0	0	0		0
Human "O"		HA	4	4	4	4	4	4	4	0	0	0		0
		HL	4	4	3	2	1	±	0	0	0	0		0

* Figures according to a scale ranging from 0 to 4, denote (HA) degree of hemagglutination and (HL) degree of hemolysis.

importance in reading the hemolysin (HL) tests, as the figures given were arrived at by direct comparison with the control tubes used as standards of the degree of hemolysis present. In a 1:8 dilution of the original AA fluid, 100% of the hen erythrocytes were hemolyzed, only cell stroma sedimenting to the bottom of the tube. Heating at 51°C for 15 minutes lowered the hemagglutinin and hemolysin titers only slightly, but both activities were destroyed after heating at 60°C for 8 minutes. The experiments carried out at pH 6 were not wholly satisfactory. For one thing, hen erythrocytes tend to agglutinate spontaneously in the absence of virus as the pH is lowered, a phenomenon regularly observed below pH 5. Secondly, hen hemoglobin is apparently altered at pH 6, as it becomes brown. Readings of the hemolysin were made by adding a small amount of HCl to the control tubes, to render the standards comparable. With these reservations in mind, it would appear the hemagglutination and hemolysis by NDV show some decrease in titer at pH 6. Sheep and human "O" cells gave lower titers in the hemagglutination and hemolysis tests than did hen erythrocytes. Normal AA fluids gave no hemolysis.

The NDV hemolysin can be absorbed and eluted from red blood cells as indicated in Table II. In these experiments 1 cc of NDV-infected AA fluids was added to 5 cc of a 10% suspension of hen erythrocytes and the mixture centrifuged, after incubation for one hour at 4°C. All of the hemolytic and the greater part of the hemagglutinating activity of NDV was removed from the supernatant by adsorption on the erythrocytes as shown under A of Table II. The sedimented erythrocytes plus adsorbed virus were then resuspended in buffer and incubated at 37°C for 2 hours. As indicated under B of Table II the erythrocytes recovered by subsequent centrifugation were refractory to the hemagglutinating and hemolytic activity of a fresh virus suspension. Tests on the supernatant fluid using fresh erythrocytes demonstrated that NDV had eluted, for the hemagglutination titer was even higher than in the original AA fluid. This elevation of hemagglutination titer has been explained by others[3] on a sup-

TABLE II.
Adsorption and Elution of Newcastle Disease Virus (NDV) Hemolysin and Hemagglutinin from Hen Erythrocytes.

	Test	Dilutions of NDV 2	8	16	32	64	128	256	512	1024	2048	4096	8192	Buffer cont.
Original AA fluid	HA		4*	4	4	4	4	4	4	4	4	3	0	0
	HL		4	4	3	2	1+	1	±	0	0	0	0	0
A. Incubation 4°C 1 hr														
Supernate	HA		4	4	3	0	0	0	0	0	0	0	0	0
	HL	0	0	0	0	0	0	0	0	0	0	0		0
B. Resuspended RBC from (A) incubation 37°C, 2 hr			0	0	0	0	0	0	0	0	0	0		0
Eluted RBC +	HA		0	0	0	0	0	0	0	0	0			0
Fresh NDV	HL													
Eluate and fresh RBC	HA		4	4	4	4	4	4	4	4	4	4	4	0
	HL not done as eluate too colored by previous hemolysis.													

* Figures according to a scale of 0 to 4, denote (HA) degree of hemagglutination and (HL) degree of hemolysis.

position that adsorption and elution serve to break up aggregates of virus and give greater dispersion. Hemolytic activity of the supernate could not be tested, since previous hemolysis resulting from incubation at 37°C rendered it unsuitable.

Inhibition of the NDV hemolysin by specific immune sera is demonstrated in Table III. In these tests the original AA fluids, titrated as in Table I, were used in a dilution of 1:32. Paired sera from a hen experiencing a natural attack of Newcastle disease and a pair of sera from a rabbit immunized with NDV, both gave a 64 fold rise of antibody titer in the inhibition of hemolysin test. Sera of 2 rabbits immunized against influenza virus, one with the PR 8 strain and one with LEE B, and a pair of acute and convalescent phase mumps sera were without specific inhibitory action.

To determine whether the infectivity of the NDV suspension was associated with the hemolytic activity, the AA fluids were treated with 0.2% formalin and incubated over night at 4°C. The hemagglutination and hemolysis tests showed no decrease in titer when formalin treated virus was used. An infectivity titration was carried out in embryonated eggs, using an aliquot of the formalin treated suspension. The formalin treatment lowered the LD_{50} end point of the NDV suspension from 10-9.45 to 10-1. Inoculation of controls indicated that 0.2 per cent formalin in broth was not lethal for embryonated eggs.

Discussion. Although it is impossible on the basis of present data to state whether the hemolysin associated with NDV is identical with the hemagglutinin or represents a separate factor the behavior of both is more or less parallel in the above experiments. This parallelism is noteworthy in regard to adsorption and elution from red blood cells, the response to heating and to lowering of the pH, as well as in reaction to erythrocytes of different animal species and the inhibitory effect of specific immune serum. Neither activity appears to reflect the infective potency of an NDV suspension as judged by the action of formalin. The NDV hemolysin exhibits points of similarity with the hemolysin associated with mumps virus. However,

TABLE III.
Inhibition of NDV Hemolysis by Specific Immune Serum.

Source of serum	Serum phase	Dilution of NDV								Serum cont.
		16	32	64	128	256	512	1024	2048	
Hen—natural	Acute	1*	2	3	3	3	3	3	3	0
Attack with NDV	Conval.	0	0	0	0	0	1	2	3	0
Rabbit—immunized	Pre-inoc.	1	2	3	3	3	3	3	3	0
with NDV	Immune	0	0	0	0	0	1	2	3	0
Rabbits immunized	PR8-immune	1	2	3	3	3	3	3	3	0
with influenza virus	LEE B- ”	1	2	3	3	3	3	3	3	0
Mumps patient	Acute	1	2	3	3	3	3	3	3	0
	Conval.	1	2	3	3	3	3	3	3	0

* Figures denote degree of hemolysis as compared to control titration. Table II).

in other respects, the NDV hemolysin as demonstrated above differs from the description of the mumps virus hemolysin given by Morgan *et al.*[1] especially as regards the effect of heat and a lowering of the pH. Whether these differences are real or a reflection of the fact that the NDV hemolysin is more potent is not easy to determine. In our experience, it has been difficult to find a mumps virus hemolysin of sufficient potency for comparative tests.

The fact that hemolysins are associated with both NDV and mumps virus, but not with influenza virus,[1] is of interest in indicating a possible affinity of mumps and Newcastle viruses, as first suggested by Burnet[3,4] in his work on "receptor gradients." Recent demonstration of serologic relationships between NDV and mumps virus,[5] evidenced by the presence of neutralizing and antihemagglutinating antibodies in nearly half of patients convalescent from mumps, lends further support to this hypothesis.

Summary. (1) A hemolysin associated with the virus of Newcastle disease is described. (2) The activities of this hemolysin paralleled those of the NDV hemagglutinin in regard to adsorption and elution from hen erythrocytes, inhibition by specific immune serum, inactivation by moderate heating, and in its behavior with erythrocytes of different animal species. (3) A suggestion is made that similarities in behavior between the hemolysin associated with NDV and that associated with mumps virus are further indication of possible relationship between the 2 viral agents.

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[3] Enders, J. F., Levens, J. H., and Robbins, F. C., personal communication.

[4] Burnet, F. M., *Australian J. Sci.*, 1945, **8**, 81.

[5] Jungherr, E., Luginbuhl, R. E., and Kilham, L., to be published.

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17081. Effect of Pregnancy on the Rat Ovary Transplanted to the Spleen.*

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The response of the ovary of rats to endogenous hormones, when it is placed in the portal circulation by means of intrasplenic transplantation, differs greatly from that of the ovary that is in its normal position. If the animal is a castrate, the intrasplenic ovary shows a continuous formation of new follicles which luteinize. The corpora lutea do not involute, and from them a neoplasm may ultimately develop.[1,2] If the animal is a unilateral castrate, the intrasplenic ovary atrophies after a period of 24 days, but this atrophic ovary assumes active growth as soon as the normal ovary is removed.[3] It is probable that when one ovary is intact and the other is transplanted to the spleen both are receiving the same hormonal influences through the systemic circulation. The normal ovary shows the compensatory hypertrophy that customarily follows unilateral castration, while the intrasplenic ovary simultaneously is undergoing severe atrophy. That the splenic pulp does not interfere with growth is proved by the appearance of follicles and corpora lutea in the intrasplenic ovary and its progressive enlargement, immediately following the removal of the normal ovary.[3]

The present investigation is a related study on the effect of the endogenous chorionic gonadotrophic hormones of the rat on the normal and on the intrasplenic ovary. Young adult female rats of the Long-Evans strain were employed. The left ovary was removed under ether anaesthesia through a lumbar approach and cleaned of fat and the tube. The spleen was delivered into the wound, and the left ovary was placed in a pocket made by incising the splenic capsule. After an interval of from 40 to 50 days, the rats during estrus were placed with males, and all became pregnant. Six animals were sacrificed as soon as possible after the litter was delivered; nine additional animals were permitted to live between 9 and 60 days after the termination of pregnancy. Another group of nine animals was ovariectomized as soon as possible after the litter was delivered and subsequently three of these were sacrificed on each of the following days after the birth of the litter: 10, 31, and 62.

Pregnancy had occurred only in the right horn of the uterus of each animal. The litters were composed of normal young which were removed from the mother at birth. In the group ovariectomized post partum, the right ovary was removed through a right lumbar incision, and the spleen was carefully examined for adhesions between it and the parietal abdominal wall or other organs. A complete autopsy was performed on each animal. The tissues were prepared in the usual manner for histologic study and multiple sections at several levels of the transplanted ovary were studied.

The 6 animals that were sacrificed at the termination of pregnancy showed a slight increase in the size of the transplant. Histologic examination revealed that this was due to an increase in the size and number of follicles. Many of the follicles were large and contained an ovum that was compactly surrounded by as many as ten rows of follicular cells. A single cross section through a transplant often contained as many as 10 to 15 follicles of varying size. In one transplant, there was a single small corpus luteum; serial sections of the remaining transplants did not disclose a corpus luteum. The control

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* This investigation was supported in part by a research grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and in part by a grant from the United States Vitamin Corporation.

[1] Biskind, M. S., and Biskind, G. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 176.

[2] Biskind, G. R., and Biskind, M. S., *Am. J. Clin. Path.*, in press.

[3] Biskind, G. R., and Biskind, M. S., *Science*, 1948, **108**, 137.

transplants in non-pregnant animals rarely contained as many as 5 minute atrophic follicles.[3]

Animals sacrificed at intervals after the termination of the pregnancy showed that the transplants decreased in size and became atrophic. In 7 instances, they resembled the transplants in the animals with one normally situated and one intrasplenic ovary.[3] The ovarian stroma of each of these groups was composed of clusters of small cells that may have been thecal cells. Small follicles were scattered in the stroma. In 2 transplants small corpora lutea were present.

The animals in the last group had one ovary removed at the termination of pregnancy and subsequently were sacrificed at the intervals mentioned. The intrasplenic ovary grew rapidly and increased in size as early as ten days after the operative procedure. It was composed of developing follicles and corpora lutea. At 31 and 62 days after ovariectomy, some ovaries seemed larger than the corresponding intrasplenic ones described previously.[2] It is apparent that when the normally situated ovary is removed, the hormonal factors originally described are permitted to influence the follicular and luteal growth. In this instance there may be a slightly greater degree of growth of the intrasplenic transplant because of the extent of follicular growth during pregnancy.

The results suggest that in pregnant rats there is a decided difference in the response of the intrasplenic compared to the normal ovary. In the non-pregnant rat, the normal ovary is hypertrophied, the intrasplenic ovary is atrophic. Pregnancy produced the usual large corpora lutea in the hypertrophied normally situated ovary, follicular growth in the intrasplenic ovary of 5 rats, and corpora lutea in 1 rat. Several hypotheses can be adduced to account for these unusual responses of the same gonadal tissues in different sites in the body. The action of the liver in inactivating the estrogenic hormones elaborated by the intrasplenic ovary may play a role. Consideration must be given to the effect of the estrogen secreted into the systemic circulation by the normally placed ovary and its probable limitation of growth on the intrasplenic ovary, either directly or by influencing the hypophysis. In addition, there is an increase in estrogens as well as in progesterone during pregnancy which may act on the intrasplenic ovary. The production of follicular growth only in the intrasplenic ovary in five out of six animals may be due to the increased amounts of chorionic gonadotrophins from the placenta, or to increased hypophyseal stimulation. Further studies to elucidate this unusual effect are being performed.

Summary. In 24 rats the left ovary was transplanted to the spleen, and between 40 and 50 days later they were permitted to become pregnant. Six animals were sacrificed at the termination of pregnancy, and in all the intrasplenic ovary showed distinct proliferation of new follicles, and only one of these in addition contained a corpus luteum. Nine animals were sacrificed at intervals up to 60 days post partum, and in these the intrasplenic ovary showed progressive atrophy. In another series, the normal right ovary was removed at the termination of pregnancy and groups of 3 animals were sacrificed 10, 31, and 62 days later. The intrasplenic ovary of this series showed progressive enlargement with the continuous formation of new follicles and corpora lutea, and there was no evidence of involution of the latter. In this experiment, the gonadotrophic hormones of pregnant rats produced primarily follicular activity in the intrasplenic ovary, while simultaneously in the normally situated ovary corpora lutea were formed and maintained.

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17082. By-passing the Right Ventricle.*

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A comparative analysis of the physiology of pulmonary and systemic pressures of the several classes of vertebrates indicated that the vascular tree of respiratory organs is a circuit of very low resistance.[1] The pulmonary arterial pressure was found to be of the order of 25 ± 10 mm Hg in fish, amphibiae, reptiles, mammals and birds, while the systemic pressures varied according to class and body temperature.[1,2] Consideration of these data suggested that the *vis a tergo* for pumping the venous blood through the lungs might be supplied adequately by the venous pressure. Further, in the presence of a congenital or acquired stenosis of the tricuspid or pulmonary orifice, an atrio-pulmonary shunt by-passing the right ventricle might provide a means for complete oxygenation of the entire venous return. Experiments were done on acute and chronic preparations to demonstrate the feasibility of this procedure.

Methods and Results. Acute experiments were carried out on 5 dogs anesthetized with pentobarbital. A cannula was placed in the left atrial appendage and connected with a modified Ludwig Stromuhr. The outflow from the stromuhr was via a cannula tied into the pulmonary artery so that the blood would flow in the direction of the lungs. The pulmonary artery was then tied at its origin. In this way blood was shunted from the right auricle directly to the pulmonary artery without passing through the right ventricle. Pontamine-fast Pink BL (150 mg/kilo) or heparin were used as anticoagulants.

On connecting the stromuhr, blood was seen to flow directly through the glass system. The venous pressure, measured in the right auricle, was found to be 9 to 14 cm of saline. On tieing the origin of the pulmonary artery, the right ventricle ballooned out but the dilation regressed as the experiment continued. The preparations lasted up to one hour.

Chronic experiments were carried out in 5 dogs. Under pentobarbital anesthesia and using artificial erspiration via an untratracheal catheter, the thorax was opened at the third intercostal space. The pericardium was opened via a longitudinal incision, taking care to avoid the phrenic nerve. The right atrial appendix was clamped by a nontraumatic, specially designed C-shaped forceps.‡ A second C-forceps was used to occlude about 3 cm of the right aspect of the main pulmonary artery parallel to its trunk. Using 5.0 silk on atraumatic needles, the atrial appendage was brought into approximation and anastomosed to the clamped portion of the pulmonary artery. Minimal bleeding was seen on removal of the C-clamps. The pericardium was closed only partially to prevent pericardial tamponade. The thorax was closed with linen ligatures.

The animals showed prompt recovery from the operative procedure. A loud blowing systolic murmur was heard over the entire thorax, loudest at the apex. Venous pressures were found to be about 7 cm of saline. Fluoroscopy indicated a heart size within normal limits. Dogs with these atrio-pulmonary artery anastomoses have been observed for periods up to two months. They show no notable disability. Systemic arterial blood pressures recorded with the Hamilton manometer were about 180/100 mm Hg.

In one dog, after the anastomosis as described above was completed, the origin of the pulmonary artery was tied off almost completely. This procedure tended to kink the anastomo-

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* Aided by the A. D. Nast Fund for Cardiovascular Research.

† This department is supported in part by the Michael Reese Research Foundation.

[1] Rodbard, S., Brown, F., and Katz, L. N., *Am. Heart J.*, in press.

[2] Rodbard, S., *Science*, 1948, **108**, 413.

‡ Machined by Mr. Emil Tiger of the Michael Reese Hospital Medical Research Institute Workshop.

sis. The right ventricle dilated and irregular heart action was seen for several minutes. A loud systolic murmur was heard post-operatively. The dog was well for 12 hours, but was found dead the next morning. Post mortem examination revealed a patent functioning anastomosis of 1 cm diameter.

Discussion. The acute experiments described above demonstrate that the venous pressure may operate as *vis a tergo* to pump at least some of the venous return through the lungs without benefit of right ventricular action. This illustrates anew the remarkably low resistance of the pulmonary circuit. Obstruction to outflow through the pulmonary artery results in a momentary damming of blood in the great veins until the venous pressure rises to levels of about 12 cm saline. A transmission to the venous presure of some of the pulse from the distended right ventricle continues because of the resulting equivalent of a functional tricuspid regurgitation. However, it is unlikely that this participates in the development of an increased pressure in the right auricle because of the large distensible venous reservoir in free communication with the atrium.

In the chronic experiments with production of the atrio-pulmonary shunt, a functional tricuspid regurgitation is produced because of the flow of some of the output of the right ventricle via the pulmonary artery anastomosis to the right atrium. The amount of this regurgitation has not been determined. Under normal circumstances the anastomosis results in a flow from ventricle to pulmonary artery to atrium, especially during the heightened pressure of ventricular systole. The possibility must be considered that some blood passes from atrium to pulmonary artery during diastole when the pulmonary diastolic pressure is low, and especially during atrial systole before ventricular contraction occurs. Production of a stenosis or complete occlusion of the pulmonary artery central to the anastomosis forces the flow of blood from atrium directly to the pulmonary artery.

Summary. 1. An anastomosis was made between the right atrial appendage and the main pulmonary artery.

2. In acute experiments, the venous pressure was demonstrated to drive venous return through the lungs without benefit of the pumping action of the right ventricle.

3. In chronic experiments, the anastomosis acts similarly to a tricuspid insufficiency. No deleterious effects were seen in the course of two months observations on such dogs.

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17083 P. A New Imidazoline Derivative with Marked Adrenolytic Properties.

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Intense interest in efforts to combat the adrenergic predominance associated with various vascular dyscrasias has resulted in the development of numerous agents of diverse chemical structure, including the hydrochloride of 2- [N-p′-tolyl-N-(m′-hydroxyphenyl) - aminomethyl] - imidazoline (C-7337).[1-3]

Toxicity. The acute LD_{50} values (20 rats per group) are as follows: Intravenous, 75 mg,* subcutaneous, 275 mg, oral, 1250 mg. A brief report of chronic toxicity studies in dogs has been made.[4]

Specific Sympathetic Blocking Effects 1.

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[1] Marxer, A., and Miescher, K., to be published.

[2] Meier, R., *New York Academy of Sciences*, 1947, in press.

[3] Meier, R., *Farmacoterapia Actual*, 1948, **44**, 84.

* All doses mg or μg/kg intravenously unless stipulated otherwise.

[4] Warren, M. R., Woodbury, R. A., and Trapold, J. H., *Fed. Proc.*, 1949, **8**, 343.

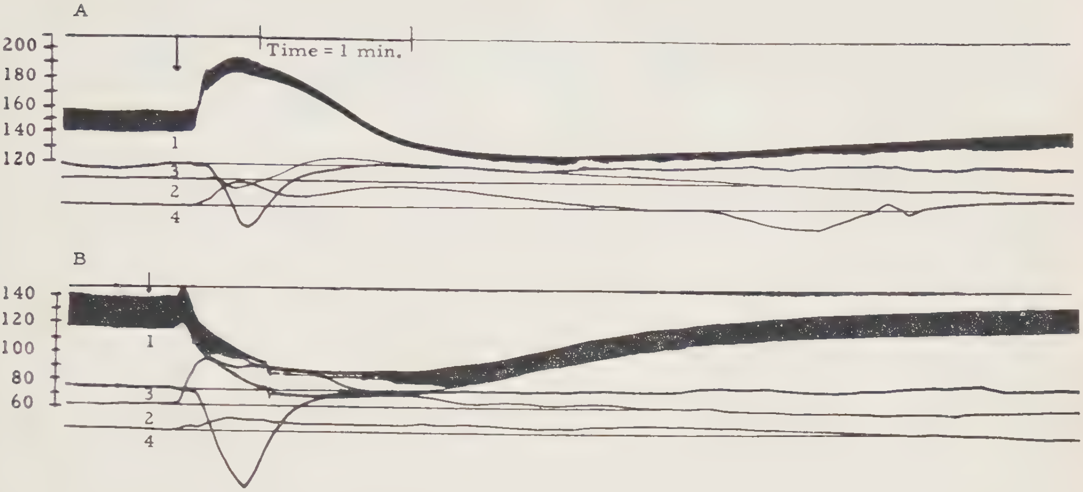

FIG. 1.

A. Cat under Dial anesthesia (70 mg/kg). The effects of 5 μg epinephrine intravenously (injected at arrow) are demonstrated on 1) blood pressure, 2) flow in mesenteric artery, 3) flow in renal artery, and 4) flow in femoral artery. Flow rates were determined with the Rein Thermostromuhr. B. Same animal 30 min. after 1 mg C-7337 subcutaneously. Note chief effect is "epinephrine reversal." Flow rates qualitatively the same as in A. Time = 1 minute.

In mice the LD_{100} of epinephrine subcutaneously injected is 7.5 mg. C-7337, 0.03 mg injected subcutaneously half an hour before the epinephrine, permitted a 20% survival; 0.1 mg administered in the same manner protected 40% against the lethal dose of epinephrine; after C 0.3 mg there was 100% survival.

2. The rise of blood pressure after epinephrine (0.1-10 μg) was diminished in cats, dogs and rabbits (Dial anesthesia) by doses of 0.01-0.03 mg C-7337 intravenously or subcutaneously and completely inhibited in cats and dogs by 0.1 mg intravenously or subcutaneously. The typical "epinephrine reversal" was produced in cats and dogs by doses of 0.1-1.0 mg intravenously or subcutaneously (Fig. 1). The epinephrine reversal was also observed in rats and guinea pigs and in the spinal cat. Despite this inversion, the typical effect of epinephrine on blood flow in the renal and femoral arteries remained qualitatively unchanged (Fig. 1).

3. The sustained vasoconstriction produced by constant arterial perfusion of the posterior extremities of the rabbit with epinephrine (10^{-7}) was decreased 21% by 10^{-8} C-7337, 50% by 10^{-7} and 90-100% by 10^{-6} (Fig. 2).

4. The contractile action of epinephrine (2 μg) on the nongravid rabbit uterus *in vivo* was almost entirely obliterated by 100 μg of C-7337.

5. The relaxing effect of epinephrine (5×10^{-7}) on the isolated ileum of the rabbit was diminished approximately 50% by 5×10^{-7} C-7337 and about 65% by 5×10^{-6} C-7337.

6. The minimal doses of C-7337 required to eliminate salivation in the cat were 50 μg for adrenolysis (3 μg epinephrine) and 700 μg for sympatholysis (10 second faradization of cervical sympathetic nerve).

The adrenolytic effect of C-7337 (50 μg) against 3 μg of epinephrine as a sialogogue was complete, but this blocking effect could be partially broken through by 10 or 15 μg of epinephrine.

7. As a rule higher doses of C-7337 were required to produce an adrenolytic and sympatholytic effect upon the nictitating membrane than upon blood pressure and the salivary gland. The retraction of the nictitating membrane produced by epinephrine (10 μg) was almost completely inhibited by 1.0 mg C-7337; after 10 seconds of faradization it was eliminated by 17.7 mg C-7337.

8. Epinephrine hyperglycemia (50 μg) in rabbits was not prevented with doses of 0.15

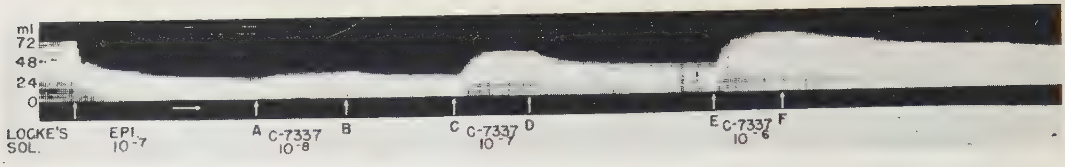

FIG. 2.

Rabbit, hind limbs continuously perfused with Locke's solution containing epinephrine, 10^{-7}. Each vertical line represents an interval of 10 seconds during which the varying rates of flow were registered in milliliters; normal flow was approximately 75 ml per 10 seconds whereas epinephrine, 10^{-7}, reduced this rate to approximately 25 to 30 ml per 10 seconds. From A to B, perfusion with C-7337, 10^{-8}; from C to D, C-7337, 10^{-7}; from E to F, C-7337, 10^{-6} concentration. Note the increasing antagonism of epinephrine's vasoconstrictor action following gradually increased concentrations of C-7337.

to 15 mg C-7337 subcutaneously.

9. The adrenolytic effects of adequate amounts of C-7337 (1 to 3 mg) on the vasopressor response to epinephrine (2 μg) were observed also after absorption following ileal intubation in the cat and dog.

Miscellaneous Features. 1. C-7337 was invariably hypotensive in all species examined (rabbit, cat, dog, rat, guinea pig) and in any dose which produced adrenolytic effects after the conventional, reasonably rapid injection. The hypotensive effect was more marked with increasing dosage and endured—depending on the dose employed—for 2 to 3 hours or more. After intravenous injection the fall in blood pressure was more pronounced than after subcutaneous injection.

2. C-7337 in a concentration of 10^{-6} decreased the coronary flow of the isolated perfused heart of the rabbit 25%, the rate 8% and the amplitude 17%. The corresponding reductions for the feline heart were 30%, 12% and 13%.

3. The isolated vessels of the hind limb of the rabbit showed no reaction to concentrations of C-7337 up to 10^{-5}.

4. No direct myotropic stimulating effect was observed in respect to the bronchi of the rabbit, nictitating membrane of the cat, isolated ilea of the rabbit and guinea pig or intact ileum of the dog. As opposed to the majority of adrenolytic substances, C-7337 has no contractile action upon the isolated uterus of the guinea pig or the uterus *in vivo* of the rabbit.

5. No sialogogic (cholinergic) effect was evident in the cat even with a dose of 27.5 mg of C-7337, whereas faradization of the chorda tympani nerve consistently resulted in salivation.

6. Warburg studies have indicated that C-7337 did not appreciably interfere with feline cardiac respiration until concentrations approaching 500 μg/ml had been reached. At this concentration there was a 12.5% decrease in cumulative oxygen consumption after 4 hours.[5]

Conclusions. The compound 2- [N-p′-tolyl-N-(m′-hydroxyphenyl) -aminomethyl] -imidazoline HCl, (C-7337), is a potent adrenolytic agent but much less active as a sympatholytic drug. It is effective orally.[6]

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[5] Herrold, E. A., Cameron, A., Earl, A., Roth, F., Smith, J., Smith, N., Sorensen, E., and Craver, B. N., *Fed. Proc.*, 1949, **8**, 302.

[6] Grimson, K., Chittum, J. R., and Longino, F. H., *Fed. Proc.*, 1949, **8**, 61.

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17084. Effects of Desoxypyridoxine and Vitamin B_6 on Development of the Chick Embryo.*

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The usefulness of metabolite analogs in nutritional and metabolic studies has been demonstrated by a number of workers. These studies have been summarizd by several authors.[1-3] It appeared logical, therefore, that vitamin analogs might be used in studies dealing with chemical embryology, and as an approach to this problem, a study of the effects of desoxypyridoxine (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine) on the development of the chick embryo was begun. Ott[4] has shown that this analog is markedly inhibitory to growth of chicks on diets which contain amounts of pyridoxine which limit growth, and that its effects are overcome by administration of additional pyridoxine.

While these studies were in progress, Ackermann and Taylor[5] reported the application of this technic to the study of the metabolism of chick embryos. The analog used by these workers, 3-acetylpyridine, was found to inhibit embryonic development and this inhibition could be prevented by nicotinamide. Nicotinic acid and tryptophan were much less active reversing agents.

Experimental. The procedure used in injecting the various compounds into the eggs was as follows: With the egg standing on its small end, the large end was swabbed with ethanol. A small hole was then drilled into the egg through which the compounds were injected. A tuberculin syringe and a No. 26 gauge needle approximately 1.5 cm in length were used for this purpose. The needle was inserted into the egg at such an angle that the solutions injected were deposited in the albumen in close proximity to the yolk. It is possible that occasionally the yolk was pierced by the needle but the data presented show that control injections of water did not result in a significant decrease in hatchability. In the first experiments the compounds were dissolved at concentrations which required injection of 0.2 cc of solution. This amount of water injected into eggs prior to incubation resulted in some decrease in hatchability. One-tenth cc appeared harmless, however, and in further experiments this volume of solution was used. For the injection of higher levels of the compounds at six days of incubation, volumes up to 0.5 cc could be used without harm. After the desired solutions were injected the hole was sealed with collodion and the eggs were immediately set in an incubator.

The eggs were candled at irregular intervals and the infertile eggs and dead embryos were removed. All eggs were broken out and examined to determine fertility and approximate age at death of the embryo. Any gross abnormalities were also recorded.

Results. The data of Table I show the effect of the injection of desoxypyridoxine prior to incubation on embryonic development. Approximately 500-1000 μg of the analog caused 100% mortality of the embryos. This inhibitory effect of the analog was largely prevented by simultaneous injection of 1.0 mg of pyridoxal hydrochloride. The "toxic" effect of desoxypyridoxine at these levels thus appears to result from its interference with normal utilization of vitamin B_6, and can be prevented by supplying additional quantities

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[1] Roblin, R. O., Jr., *Chem. Rev.*, 1946, **38**, 255.

[2] Woolley, D. W., *Advances in Enzymol.*, 1946, **6**, 129.

[3] Wright, L. D., *J. Am. Dietet. Assn.*, 1947, **23**, 289.

[4] Ott, W. H., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 125.

[5] Ackermann, W. W., and Taylor, A., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 449.

TABLE I.
Effect of Desoxypyridoxine on Hatchability of Eggs.

Substance injected	Amt injected, µg	% Hatchability* 0.2 cc solutions	 0.1 cc solutions
None		74	95
Water (H_2O)		35	90
Desoxypyridoxine	200	29	
,,	500	11	21
,,	1000	0	0
,, + Pyridoxal · HCl	1000 1000	13	83

* Not less than 14 eggs per group.

TABLE II.
Effect of Injection of Desoxypyridoxine at Different Stages of Incubation of Hatchability.

Time of injection†	% Hatchability* Water	 500 µg Desoxypyridoxine	 Water	 500 µg Desoxypyridoxine	 Water	 1000 µg Desoxypyridoxine
0 days	88	24	87	14		
68 hr						5
72 ,,	94	72	86	64	80	4
86 ,,						53
92 ,,						79
6 days	93	89	91	91		
9 ,,	95	88				
12 ,,	95	89				
15 ,,	100	95				
Control (uninj.)	95		96		84	

* 15-23 eggs per group.
† 0.1 cc of water or solution of inhibitor injected in all cases.

of the vitamin. The result demonstrates that vitamin B_6 is highly essential for normal embryonic development. It was observed that most of the embryos died at 2 to 4 days of development.

The effects of injecting desoxypyridoxine at different stages of incubation on embryonic development are shown in Table II. Whereas 500 µg of the analog injected prior to setting resulted in approximately 75% mortality of the embryos, a similar quantity injected at 3 or more days of incubation was non-toxic. Injecting 1000 µg at 68 or 72 hours of incubation resulted in approximately 95% embryonic mortality. This inhibitory effect was almost immediate since the bulk of the embryos were recorded as dying at 3 or 4 days of development. However, this same amount injected at 86 or 92 hours had little, if any, effect on embryonic development. The inhibitory effects of this analog are thus most marked during the first 3 to 4 days of embryonic development; subsequent to this time, the compound has greatly reduced toxicity.

In view of this fact, the toxic level of the analog at 6 days of incubation was determined. In a preliminary experiment, 5 mg of the analog proved almost completely inhibitory at this time, but this inhibition could not be overcome by simultaneous injection of any of the 3 forms of vitamin B_6. Further experiments, summarized in Table III, indicate that pyridoxal hydrochloride is also toxic at these high levels, while pyridoxine hydrochloride is somewhat less toxic and pyridoxamine dihydrochloride proved non-toxic at the highest level tested (10 mg per egg). Inhibition at this period by these high concentrations of desoxypyridoxine, therefore, is not specifically related to an interference in utilization of vitamin B_6 for essential metabolic purposes, as is the inhibition by smaller amounts of

TABLE III.
Comparative Toxicities of Desoxypyridoxine, Pyridoxal, Pyridoxamine, and Pyridoxine When Injected at 6 Days of Incubation.

Mg of compound injected	% Hatchability†			
	Desoxypyridoxine	Pyridoxal hydrochloride	Pyridoxamine dihydrochloride	Pyridoxine hydrochloride
Uninj. control	100	100	100	100
Inj. control*	70	70	70	70
1.0	80	90	70	100
2.5	70	50	50	90
5.0	0	20	90	80
10.0	0	0	90	10

* 0.5 cc of water or solutions injected in all cases.
† 10 fertile eggs per group.

TABLE IV.
Comparative Potencies of Pyridoxal, Pyridoxamine, and Pyridoxine in Overcoming the Inhibitory Action of Desoxypyridoxine.

Substance injected*	Amt injected, μg 0	2	5	10	20	50	70	100	1000
	% hatchability†								
Desoxypyridoxine									0
Desoxypyridoxine, 1000 μg									
+ Pyridoxal · HCl		0	0	0	18	30	56	63	47
Pyridoxamine · 2HCl		0	14	27	45	71	67	71	57
Pyridoxine · HCl		0	15	50	74	71	83	62	92

* 0.1 cc of water or solution injected in all cases. Control groups run at the same time gave the following per cent hatchability: water, 75; 1000 μg pyridoxal hydrochloride, 83; 1000 μg pyridoxamine dihydrochloride, 62; 1000 μg pyridoxine hydrochloride, 60; uninjected, 83.
† All groups contained 12 fertile eggs or over; at critical levels (20 and 50 μg of vitamin plus 1000 μg of desoxypyridoxine) 23 to 40 fertile eggs were used per group.

desoxypyridoxine which occurs when this compound is injected before the start of incubation.

It has been shown[6] that pyridoxal and pyridoxamine are the predominant forms of vitamin B_6 present in both the egg and the chick; pyridoxine, if present at all, occurs only in relatively small amounts. It was of interest, therefore, to compare the relative activities of these three compounds in counteracting the inhibitory action of desoxypyridoxine. Results of such an experiment are summarized in Table IV. The ratios of vitamin to inhibitor which permitted approximately 50% of the embryos to produce live chicks were for pyridoxal hydrochloride 1:20; for pyridoxamine dihydrochloride 1:50; and for pyridoxine hydrochloride 1:100.

Discussion. These experiments show clearly that development of the chick embryo is inhibited by the injection of desoxypyridoxine when this is made prior to the start of the incubation period, and that this inhibition is prevented by simultaneous injection of any of the three forms of vitamin B_6. Vitamin B_6 is an essential catalyst for a variety of reactions involved in protein metabolism, as well as in the synthesis of nicotinic acid from tryptophan. Which of these reactions first fails the embryo because of the blocking action of the inhibitor is not known. Since nicotinic acid may arise from tryptophan in metabolism and since nicotinic acid is synthesized by the chick embryo at an increasing rate as embryonic development progresses,[7] this compound was injected together with desoxypyridoxine to determine whether the inhibitor simply blocks its formation from tryptophan. Neither nicotinic acid nor its amide affected the inhibitory properties of the desoxypyridoxine. Similar negative results were obtained when DL-alanine was injected either alone or with nicotinic acid. DL-alanine was tested since Holden and Snell[8] have shown that D-alanine will replace vitamin B_6 for growth of certain microorganisms.

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[6] Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, 1948, **176**, 1157.

[7] Snell, E. E., and Quarles, E., *J. Nutrition*, 1941, **22**, 483.

Umbreit and Waddell[9] have offered an explanation for the mode of action of desoxypyridoxine which may explain our observations that the amount of desoxypyridoxine required to inhibit embryonic growth is increased when injection of the inhibitor is postponed until later in the incubation period. These workers, who used cell preparations containing tyrosine decarboxylase as their test system, showed that desoxypyridoxine exerts its inhibitory effect by being phosphorylated and then competing with pyridoxal phosphate for the apoenzyme. Desoxypyridoxine phosphate was found to be only slightly inhibitory if the pyridoxal phosphate had first been allowed to combine with the tyrosine decarboxylase protein. It is suggested, therefore, that desoxypyridoxine, when injected prior to the start of embryonic development, is phosphorylated and thus is able to compete with pyridoxal phosphate for combination with some newly-formed apoenzyme with which this coenzyme functions, and whose function is necessary for continued development of the embryo. If injection of the inhibitor is delayed beyond about three days, this apoenzyme has already been saturated with pyridoxal phosphate, thus making it impossible for desoxypyridoxine phosphate, even if formed, to exert its inhibitory action.

The toxicity of high levels of desoxypyridoxine when injected at six days of embryonic development was not due to a competitive antivitamin action of the inhibitor since it could not be prevented by any of the 3 forms of vitamin B_6. Pyridoxal hydrochloride proved to be toxic at similarly high levels while pyridoxine hydrochloride was somewhat less toxic and pyridoxamine dihydrochloride was nontoxic at the highest level tested.

The role of vitamin B_6 in development of the chick embryo is not readily determined directly, since hens fed diets deficient in this vitamin stop laying before sufficient eggs are obtained to determine its effect on embryonic development.[10] Our experiments show that vitamin B_6 is essential for embryonic development and also that the need for this vitamin becomes critical early in embryonic growth. It is thus possible to use antimetabolites to study problems dealing with the nutrition of the avian embryo which cannot be studied directly.

Summary. Desoxypyridoxine (1000 μg) injected into eggs prior to the start of the incubation period resulted in 100% mortality of the embryos; this inhibition was prevented by simultaneous injection of any of the three forms of vitamin B_6. The ratio of vitamin to inhibitor which permitted approximately 50% of the embryos to produce live chicks was 1/20 for pyridoxal, 1/50 for pyridoxamine and 1/100 for pyridoxine.

When injected after 4 or more days of incubation, 1000 μg of desoxypyridoxine proved non-toxic. Higher levels injected at 6 days of incubation proved toxic but this toxicity could not be prevented by any of the 3 forms of vitamin B_6. A possible explanation for this observed variation in toxicity of desoxypyridoxine at different stages of incubation is offered. Pyridoxal hydrochloride and pyridoxine hydrochloride also proved toxic at high levels while pyridoxamine dihydrochloride was not toxic at the highest level tested.

Neither nicotinic acid nor its amide affected the inhibitory action of desoxypyridoxine; negative results were also obtained when DL-alanine was injected alone or with nicotinic acid.

These experiments demonstrate that vitamin B_6 becomes essential for embryonic growth very early in the incubation period.

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[8] Holden, J. T., and Snell, E. E., *J. Biol. Chem.*, 1949, **178**, 799.

[9] Umbreit, W. W., and Waddell, J. W., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 293.

[10] Cravens, W. W., Sebesta, E. E., Halpin, J. G., and Hart, E. B., *Poultry Sci.*, 1946, **25**, 80.

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17085. Failure of Human Convalescent and Hyperimmune Monkey Poliomyelitis Serums to Neutralize "Egg Adapted" Lansing C (M) Variant.

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Enright and Schultz[1] reported the successful propagation of the Lansing C (M) strain of poliomyelitis virus in the embryonated hen's egg. Their conclusion was based on the production of typical lesions and paralysis in monkeys with early egg-passage material only. In addition, neutralization was accomplished by anti C (M) rabbit serum, but not by antiserums against the murine SK and Theilers GD VII strains, although some cross-reaction was observed with the former. Unreported observations by Schultz[2] have since indicated that the so-called Lansing variant is more closely related to the murine SK strain than to the original Lansing C (M) virus. More recently, Powell and Jamieson[3] have reported the isolation of an apparently similar variant in embryonated eggs. They subsequently proved by mouse protection tests that it was serologically distinct from the original Lansing CM virus and similar to the MM and murine SK strains. Previous unsuccessful or questionable attempts to propagate the Lansing strain to eggs have been reported by Riordan and Sa-Fleitas[4] and Gard.[5]

The egg-adapted variant mentioned above was received from Dr. Edwin W. Schultz in 1948 and readily multiplied in chick embryos.[1] The range of infectivity for mice by the intracerebral route was between 10^{-5} and 10^{-7}. Preparations from the first and subsequent passages made in this laboratory were also infectious for mice by the intraperitoneal, subcutaneous, intravenous, intranasal, and occasionally by the oral route. Seven and a half times the intracerebral dose was necessary for infection intraperitoneally. The regularity with which paralysis occurred following intraperitoneal injection suggested the possibility of using this means for human serum neutralization tests. For this purpose, 58 specimens of serum from convalescent cases of poliomyelitis were studied. Of these, 33 were taken within 6 months after the onset of the disease, 10 between 6 months and 5 years, and 11 between 5 and 13 years. The time of onset in the other cases was unknown. Immune guinea pig serum was prepared against the Schultz variant for use as a control.

Methods. Neutralization tests were performed in the following manner. The virus was prepared so that 0.1 ml contained 50 LD_{50} as titrated by the intraperitoneal route. The convalescent serums were diluted either 1:5 or 1:10, and mixed with equal volumes of the virus suspension. Extract broth was used as the diluent in both cases. The mixtures were incubated in a 37°C water-bath for 2 hours, and 0.2 ml inoculated intraperitoneally into 3-week-old Swiss mice. Controls with homologous immune serum, normal guinea pig serum, and in some cases monkey serum were run simultaneously with each series. Six to 8 mice were used for each serum tested. During the course of experiments, three separate lots of egg-passage material were utilized, each being previously standardized for the LD_{50} dose. Animals dying within the first 24 hours following injection were discarded; death or paralysis after this period was taken as the end-point and usually occurred during the first week. All experiments were terminated at the end of 2 weeks. Irregular results in the control animals were obtained occasionally. These were found to be due to the use of animals older than four weeks, rather than to deterioration of the virus, which was stored at −6°C.

Results. A total of 58 human convalescent poliomyelitis serums were tested. Of these, 56 showed no neutralization whatsoever. Only 2 gave slight but definite protection. With 50 LD_{50} of virus, the 50% end-point with these serums did not exceed 10^{-1}. In several experiments complete protection was never obtained with 50 LD_{50} of virus, but in two experiments complete protection against 25 LD_{50} was provided by a 1:5 dilution of the serums. Of interest is the fact that the serum of one patient was obtained 13 years after the clinical disease, whereas the other was drawn 3 weeks after the onset of paralysis.

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[1] Enright, J. B., and Schultz, E. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 541.

[2] Schultz, E. W., personal communication.

[3] Powell, H. M., and Jamieson, W. A., *J. Infect. Diseases,* 1948, **83**, 238.

[4] Riordan, J. T., and Sa-Fleitas, M. J., *J. Immunol.,* 1947, **56**, 263.

[5] Gard, S., *Nature,* 1943, **152**, 660.

Similar tests were carried out using potent hyperimmune monkey serums[*] against the Lansing, Kotter (Brunhilde group) and Brockman strains of human poliomyelitis virus. None of these serums protected against the Schultz variant. Experiments in which 10, 25, and 50 LD_{50} of virus were used, together with a 1:5 serum dilution, showed no significant difference between the hyperimmune and pooled normal monkey serums. On the other hand, the "variant" immune guinea pig serum completely protected against 50 LD_{50} of MM virus.[†]

Summary. The egg-adapted variant originally obtained by Enright and Schultz has not proved to be of value as a means of measuring antibody response in serums from hyperimmune monkeys and convalescent human poliomyelitis cases. The results support the conclusions of Schultz, and of Powell and Jamieson that the egg-adapted variant is serologically related to the MM-SK group of murine encephalomyelitis viruses, rather than to human and monkey-adapted strains.

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[*] These antiserums were obtained through the courtesy of Dr. I. M. Morgan and Dr. J. F. Kessel.

[†] Obtained from Dr. C. A. Evans.

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17086. Electron Microscopy of Tissue Cultures Infected with the Virus of Eastern Equine Encephalomyelitis.[*]

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During the past 2 years we have been studying the growth of several viruses in tissue culture with particular emphasis on the use of the electron microscope. The cellular destruction and overwhelming growth of the virus of eastern equine encephalomyelitis are here reported.

Successful electron microscopy of virus infected cells has been limited to studies on "virus induced" tumors[1,2] and has not been applied to small rapidly multiplying viruses. Our technic is essentially that of Porter[3] except that cells are usually fixed for only ten minutes with osmic acid vapor.[4]

Chick embryo tissue was cultured for 6

TABLE I.
Equine Encephalomyelitis in Tissue Culture. Titration on 10-day Embryos of Fluid Removed from Tissue Cultures.

Exp. No.	Original inoculum	Titer of virus in tissue culture fluid
1	10^{-2}	$10^{-5.5}$
2	10^{-3}	$10^{-6.5}+$
	10^{-3}	$10^{-6.5}+$
3	10^{-4}	10^{-7}

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[*] Supported in part by grants-in-aid from the National Institutes of Health, U. S. Public Health Service.

[1] Claude, A., Porter, K. R., and Pickels, E. G., *Cancer Research*, 1947, **7**, No. 7.

[2] Porter, K. R., and Thompson, H. P., *J. Exp. Med.*, 1948, **88**, 15.

[3] Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.

[4] Bang, F. B., and Gey, G. O., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 86.

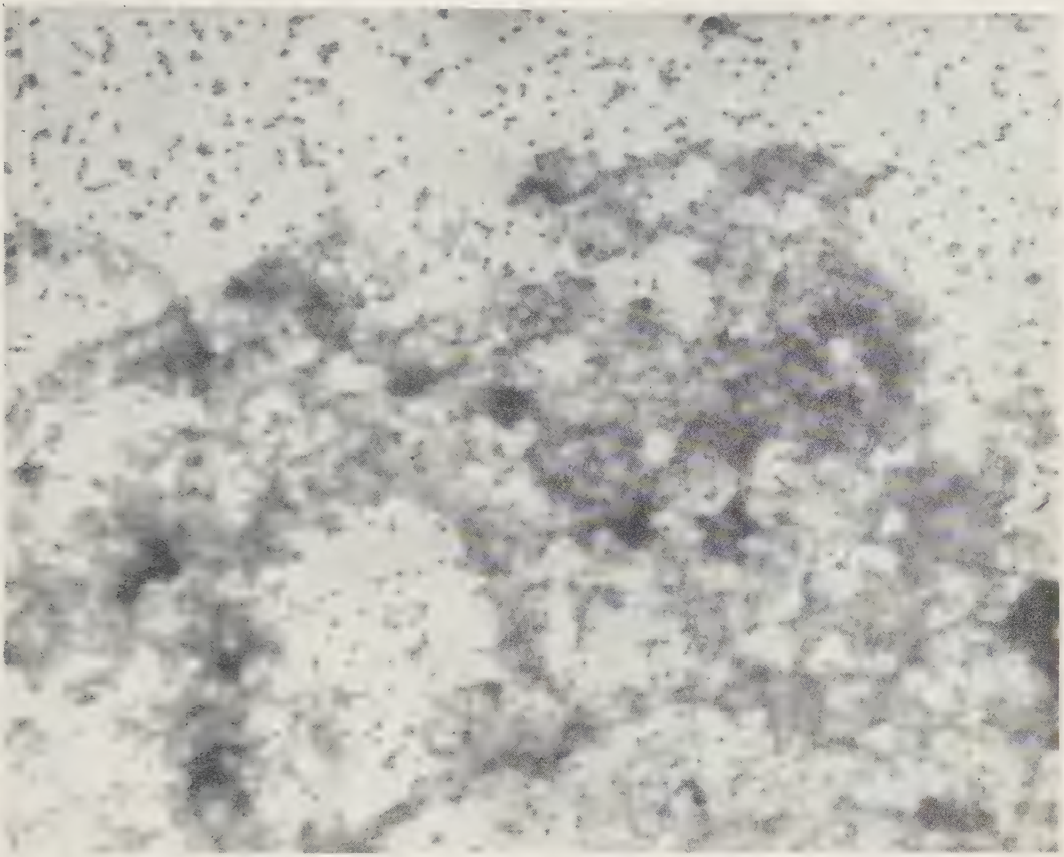

FIG. 1. Preparation of cell destroyed by Eastern equine encephalomyelitis virus. × 15,734. Fixed at 37°C with 2% osmic acid vapor for 10 minutes.

days with one transfer in roller tubes[5] in a completely homologous medium composed of chicken plasma and serum and chick embryo extract. Pieces of this tissue were then exposed to a 10^{2} dilution of the virus. A saline extract of embryos infected with the fourth embryo passage of a standard guinea pig passage of eastern equine encephalomyelitis was used as source of virus. This tissue explant was then placed in a special chamber. This was formed by drilling the center out of a metal plate and sandwiching it between 2 formvar coated glass slides. The medium consisted of 20% chicken serum. Within the next 24 hours of incubation at a temperature of 37°C, the cells had grown out poorly and were already beginning to be destroyed. The cells were fixed by exposure to osmic acid vapor and were either examined directly or were first coated with chromium to sharpen the image. The fluid taken from the tissue culture chamber taken just before osmic acid fixation of the preparation shows a high titer of infectivity for the chick embryo. (Table I). The titers obtained indicate that considerable multiplication of the virus has taken place. This is indicated by the following figures. An inoculum of chick embryo extract would at the most titer $10^{-3.5}$ This inoculum was used in our experiments at a 10^{-4} dilution which was further diluted at least 10^{-1}. The virus, however, was recovered regularly from the tissue culture at 10^{-6} which would be at least 100 times as much virus as was added.

Several things are notable about these pictures: (a) Many particles of uniform

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[5] Gey, G. O., *Am. J. Cancer*, 1933, **17**, 752.

[6] Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *Arch. Path.*, 1943, **36**, 167.

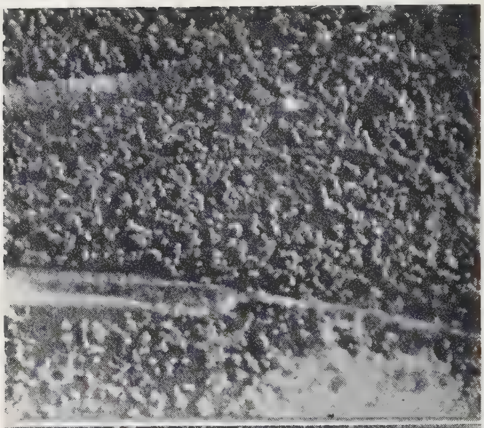

FIG. 2. (top). Chromium shadowed preparation of the same slide. Individual particles may be seen breaking off cell at bottom. × 10,300.

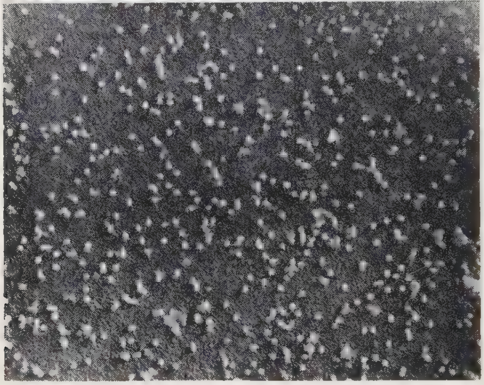

FIG. 3. (bottom). Area near a cell. Virus has been scattered over formvar screen. Dividing forms seen throughout.

size similar to those described in purified preparations[6] are associated with destroyed cells and have spilled out into the surrounding areas. The extensive cellular destruction is similar to that produced by infection of the embryo.[7] Such particles have not been seen in similar preparations of cells infected with the viruses of Newcastle disease of chickens, influenza, or mumps. (b) Examination of the pictures shows numerous pairs of particles with occasional chains and small clumps. The general appearance is much like that of a smear of ordinary diplococci. Further, it is relatively easy both in the shadowed and unshadowed pictures to trace a series of stages in the division of the pairs which suggests that multiplication may take place by simple binary fission. (c) These results make it probable that some viruses may be studied by electron microscopy of tissue culture preparations without previous purification from tissue extracts.

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[7] Bang, F. B., *J. Exp. Med.*, 1943, **77**, 337.

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17087 P. Reimplantation of Eyes in Fishes (*Bathygobius soporator*) with Recovery of Vision.*

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The early demonstration by Matthey,[1] that the grafted adult urodele eye is capable of recovering visual function has since been extensively confirmed.[2] With regard to fishes and the anuran amphibians, on the other hand, repeated failure of numerous attempts by various investigators to obtain visual recovery after eye transplantation, even in young tadpole stages, has given rise to the impression that the optic nerve of the grafted eye in these forms lacks the power for functional regeneration.[2]

Recently, however, the writer was able to show that good visual recovery can be obtained after optic nerve section in tadpole and adult frogs,[3,4] and also after eye transplantation when the transplantation is performed near the onset of metamorphosis.[4] This, plus current findings that the optic nerve of teleosts likewise is quite capable of good functional regeneration after severance[5,6] and even, in one case, after eye reimplantation,[6] prompted a further attempt to secure visual recovery after eye transplantation in fishes.

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* The experiments were conducted at the Lerner Marine Laboratory of the American Museum of Natural History, at Bimini, British West Indies. They were aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[1] Matthey, R., *Compt. rend. Soc. Biol.*, 1926, **94**, 4.

[2] Stone, L. S., *Trans. N. Y. Acad. Sci.*, 1941, **3**, 208.

Experimental. The goby was selected because of its small size and comparative hardiness to surgical trauma and low oxygen tension. The specimens were gathered during February from tide pools on North Bimini. A total of 22 animals, ranging in length from 14 to 29 mm were anesthetized in a 1.5% solution of urethane and operated upon out of water under a dissecting microscope. The left eye was first removed through a dorsal incision in the roof of the orbit. Through a similar incision in the right orbit, the right optic nerve and extraocular muscles were severed. The eyeball was then lifted out of the orbit and cut completely free from all blood vessels and other connections. Any blood clots that formed in the orbit were carefully removed with cleansing tissue before the eye was replaced. In these preliminary experiments only the right eye was reimplanted and it was always replaced in its own orbit and as nearly as possible in its original orientation. These fish, like the frog tadpole, possess a free external cornea which was left intact and which helped to hold the eye in position after its reimplantation.

After the operation the fish were placed in an aquarium with running sea water in a darkroom on the supposition that retinal metabolism might be somewhat reduced in darkness and that this might be beneficial during the early post-operative period while circulation was being reestablished. Beginning on the 6th day after operation the darkroom was lighted for 10 to 20 minutes each day for feeding the fish and for extraneous purposes. On the 21st day the fish were shifted into two aquaria in the light with standing sea water that was changed every 4 days.

When the animals were examined on the 9th day after operation, the external features of the eye appeared quite normal and eye movements were present in several cases, but there was no sign of vision at this time. When tested again on the 27th day, 2 cases displayed unmistakable evidence of visual recovery. They rose off the bottom and swam directly upward approximately 100 mm toward a small piece of bait less than 4 mm in diameter dangled on the end of a fine silk thread. By the 37th day, 7 of the 16 fish which were still alive had come to perform regularly in this manner. Similar responses were never observed in blind gobies. The accuracy with which those with vision were able to locate small stationary lures indicated that the regenerated fibers had restored their central connections in an orderly manner just as occurs after simple optic nerve section.[5] In control tests in which the bait was presented outside the glass walls of the aquarium all 7 cases responded in typical fashion attempting to reach the lure through the glass. Those which recovered vision measured in total length 16, 16, 18, 22, 24, 25, and 29 mm respectively.

Following anesthetization with urethane on the 38th day, all the heads were fixed in Bouin's solution and were later prepared for microscopic study by a modification of the Bodian Protargol method.[7] Patches of retinal degeneration, particularly in the ganglion cell and inner plexiform layers, were found in all cases but were especially marked in those that failed to recover vision. At some points all retinal layers had disintegrated. In those that recovered vision, a large regenerated optic nerve was present, the fibers of which were easily traceable into the optic tectum of the mesencephalon. Similar regenerated optic nerves were also found in 2 of the cases in which visual feeding reactions had not yet appeared at the time of sacrifice.

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[3] Sperry, R., *J. Neurophysiol.*, 1944, **7**, 57.

[4] Sperry, R., *J. Neurophysiol.*, 1945, **8**, 15.

[5] Sperry, R., *Physiol. Zool.*, 1948, **21**, 351.

[6] Rasquin, P., *Physiol. Zool.*, 1949, **22**, 131.

[7] Sperry, R., and Miner, N., *J. Comp. Neurol.*,

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17088. Influence of Cold on Blood Fibrinogen Concentration.*

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Changes of several chemical constituents of the blood are known to follow the same pattern under diverse conditions of non-specific stress. These biochemical changes have therefore been considered to be manifestations of the general adaptation syndrome. A survey of the literature suggests that perhaps variations in blood fibrinogen belong to this same group. Thus an increase in plasma fibrinogen has been reported in many non-specific stress conditions, such as infectious diseases[2] and a wide variety of chemical and physical injuries.[3,4,5]

On the other hand, a decrease in plasma fibrinogen appears to be almost always associated with specific disturbances directly related to its production, such as dietary deficiencies[2] or extensive liver damage.[5]

We therefore decided to study the blood fibrinogen changes occurring in rats exposed to cold, during the stages of alarm and resistance of the "general adaptation syndrome."

Material and Methods. Eight groups of 8 female piebald rats, weighing 130-165 g (average 145 g) were used in this experiment. All animals were put into the cold room (2-5°C) in individual cages, except 2 groups kept at room temperature as controls. All groups were maintained throughout on Purina Fox Chow, with tap water *ad libitum*. The 6 groups of rats were removed from the cold room, bled and killed, after 10, 24, 48 hours and 5, 10, 21 days respectively. During the last 24 hours of exposure all animals were fasted, but water was allowed. Blood samples were taken by carotid puncture under ether anesthesia. Blood coagulation was prevented by the use of silicone-coated syringes and needles, the samples being added to tubes containing 3 mg of potassium oxalate per ml of blood.

At the end of the experiment, one group of control rats was bled and killed without fasting, while the other group was fasted for 24 hours before bleeding.

At autopsy all the animals were carefully inspected for any detectable infection. The adrenals and thymus were removed, fixed in formalin and weighed.

The plasma fibrinogen was determined by a modification of the method of Campbell and Hanna[6] with direct nesslerization. In order to avoid the digestion in centrifuge tubes which frequently causes loss of material by bumping, we modified it as follows: the precipitated fibrinogen was quantitatively transferred with distilled water to a Folin 50 ml graduated digestion tube. The tube was then placed in an oven at 90-100°C overnight in order to evaporate the water. The following day the dry residue was digested with 1 ml of the undiluted digestion mixture. Decoloration frequently occurs following nesslerization due to the presence of an inert precipitate formed during digestion. This was prevented by diluting the digest exactly to 50 ml with distilled water, centrifuging the solution and pipetting 25 ml of the clear supernatant. The nesslerization was then performed in a 50 ml cylinder. To avoid the turbidity which occasionally occurs during the nesslerization, the diluted digest was made up to 35 ml with distilled water and the cylinder immersed in a cold bath for several minutes. Then 15 ml of the Nessler's reagent (Folin's formula) was quickly added, mixed immediately by inversion and the cylinder maintained in the water bath until read in the colorimeter. All determinations were made in duplicate.

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* This investigation was supported by a research grant from the Commonwealth Fund.

[1] Selye, H., *J. Clin. Endocrinology*, 1946, **6**, 118.

[2] Ham, T., and Curtis, F. C., *Medicina*, 1938, **17**, 413.

[3] Chanutin, A., Hortenstine, J. C., Cole, W. S., and Ludewig, S., *J. Biol. Chem.*, 1938, **123**, 247.

[4] Chanutin, A., and Ludewig, S., *J. Biol. Chem.*, 1947, **167**, 313.

[5] Foster, D. P., and Whipple, G. H., *Am. J. Physiol.*, 1922, **58**, 407.

[6] Campbell, W. R., and Hanna, M. I., *J. Biol. Chem.*, 1937, **119**, 15.

TABLE I.
Blood Fibrinogen and Adrenal and Thymus Weights During Adaptation to Cold.

No. of animals	Body wt		Time of exposure	Adrenal wt, mg	Thymus wt, mg	Plasma fibrinogen, mg/100 ml
	Initial	Final				
8*	145 ± 10.5†	—	0	27.7 ± 1.20‡	271 ± 18.0‡	252 ± 12.6‡
7	147 ± 8.9	138 ± 8.2	0	34.3 ± 1.94	163 ± 9.1	257 ± 9.3
8	146 ± 9.7	127 ± 10.3	10 hr	36.1 ± 1.50	156 ± 16.8	242 ± 15.3
7	148 ± 11.4	129 ± 9.5	1 day	35.4 ± 1.18	117 ± 9.3	191 ± 5.9
8	149 ± 12.7	127 ± 13.2	2 "	39.4 ± 1.26	108 ± 3.9	206 ± 11.1
8	148 ± 11.7	122 ± 9.0	5 "	37.7 ± 1.86	67 ± 5.2	208 ± 7.6
8	149 ± 10.0	128 ± 12.0	10 "	42.3 ± 1.95	91 ± 7.9	207 ± 6.5
8	148 ± 11.5	138 ± 16.3	21 "	43.4 ± 3.87	90 ± 14.2	208 ± 13.4

* Not fasted before fibrinogen determination.
† Standard deviations.
‡ Standard errors.

Results. Table I summarizes our results. The changes in blood fibrinogen and adrenal and thymus weights during the experimental period are also shown in Fig. 1. The values at zero time are those of the 24 hour fasted controls. The blood plasma fibrinogen decreased sharply from the control value of 257 mg to 191 mg per 100 ml in the first 24 hours. At the end of 48 hours the fibrinogen increased to 206 mg, remaining at this level until the end of the experimental period of 21 days. As can be seen from the table, fasting alone did not influence the blood fibrinogen concentration.

The weight of the adrenals increased rapidly in the first 48 hours and at a slower rate for the remainder of the cold exposure period. The thymus weights show a decrease, which was most marked at the end of the fifth day of exposure to cold, continuing approximately at the same level till the end of the experiment. The weights of these organs were used as an index showing the efficacy of the alarming stimulus. The changes observed are those constantly present in the stages of alarm and resistance of the "general adaptation syndrome".

Discussion. It seems apparent that the plasma fibrinogen does not respond in a constant manner to different types of stress as other constituents of body fluids do. In all the previously mentioned instances of blood fibrinogen increase the common feature was the tissue damage, which is known to be a stimulus for fibrinogen production. In this connection, it is noteworthy that the presence of a fibrinogen-production-stimulating substance in a turpentine-induced abscess in dogs has also been reported.[7]

In the present experiment tissue damage was not excluded. Apart from the intense catabolism indicated by the marked loss of weight, most of the animals developed severe gastric ulcers when exposed to cold and fast-

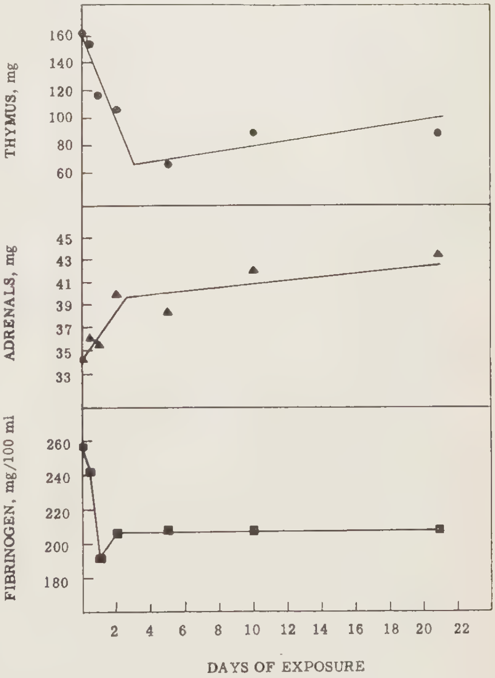

FIG. 1.

Blood fibrinogen, adrenal and thymus weights during adaptation to cold.

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[7] Homburger, F., *J. Clin. Invest.*, 1945, **24**, 43.

ing. The incidence of these ulcers was greatest at 24 hours, which coincides with the most pronounced fall in fibrinogen values. Cold, therefore, could have a specific action of depressing the fibrinogen production. It is equally possible that the plasma fibrinogen fall represents only another particular instance of the generalized protein catabolism during exposure to cold. Thus, an increased catabolism of fibrinogen, if unaccompanied by a corresponding increase in its production, appears to result in a fall of its concentration in the plasma.

Summary. In rats fasted for 24 hours before bleeding, exposure to cold causes a fall in the plasma fibrinogen concentration. The fall is maximal at the end of the first 24 hours and is followed by a slight rise to a subnormal level. This low level is maintained for as long as 21 days after beginning of cold treatment.

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17089 P. Inhibition of Multiplication of Influenza Virus by Extracts of Tea.*

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It was reported[1] previously that tannic acid inhibits the multiplication of influenza A virus in embryonated eggs. The tannic acid used was tannic acid, USP, a commercial sample, presumably extracted from nutgalls. During the course of studies designed to test the inhibitory effect of tannic acid obtained from other sources, attempts were made to extract a suitable preparation of tannic acid from tea. Preparations having certain of the characteristics of tannic acid and showing inhibitory activity have been obtained; in addition, other preparations differing, in some respects, from tannic acid and showing somewhat greater inhibitory activity than the "tannic acid" fractions also have been extracted from tea.

Materials and methods. The tea employed was a commercial sample of black tea. The PR8 strain of influenza A was used exclusively. Materials to be tested were dissolved in water, autoclaved, and neutralized with sodium hydroxide. The specified amount, in a volume of 0.5 cc, was injected into the allantoic sac of 10-day embryonated eggs and after an interval of one-half hour approximately 100 ID_{50} of virus were inoculated by the same route. Inhibition of multiplication was considered to have occurred if, after 48 hours incubation, allantoic fluid from eggs so-treated, did not agglutinate chicken erythrocytes.

Experimental. A desiccated hot water extract of tea was prepared and fractionated by the following methods: (1) by solubility or insolubility in absolute ethyl alcohol, (2) by fractional precipitation from aqueous solution with various concentrations of ethyl alcohol, (3) by precipitation from aqueous solution on addition of sufficient dilute HCl to reduce the pH to 2-3. Fractions thus obtained were desiccated and injected into groups of embryonated eggs, in amounts increasing by two-fold increments, one-half hour before inoculation of virus. The amount of each fraction per egg necessary to inhibit virus multiplication in 50 per cent of the eggs was then determined. In some instances, toxicities in terms of LD_{50} also were determined.

Inspection of Table I reveals that all fractions showed some inhibitory activity. Moreover, activity may be concentrated by removal of alcohol soluble materials, by precipitation at 88 per cent alcohol, and by precipitation at pH 2-3. Tannic acid USP is approximately twice as active but four times more toxic than

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* Aided by a grant from the United States Public Health Service.

[1] Green, R. H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 483.

TABLE I.

		Inhibitory activity		Toxicity	
Fraction No.	Description of fraction	Total No. of eggs	Amt necessary for 50% inhibition, mg per egg	Total No. of eggs	Amt necessary for 50% mortality, mg per egg
1	Hot water extract	71	0.91		
2	Alcohol soluble	43	2.00	25	7.0
3	Alcohol insoluble	27	0.66		
4	Ppt. at 64% alcohol	44	1.59		
5	Ppt. at 65 to 80% alcohol	66	0.84		
6	Ppt. at 81 to 88% alcohol	79	0.41	32	6.6
7	Soluble at 88% alcohol	36	0.92		
8	Ppt. at pH 2-3	29	0.46		
9	Soluble at pH 2-3	21	1.20		
10	Tannic acid, USP	112	0.22	32	1.6

the most active tea fraction, that precipitated at 81 to 88% alcohol (Fraction 6).

Because of its greater activity fraction 6 was chosen for more detailed study. Repeated precipitation with 88% alcohol at pH 5-6 resulted in no significant change in activity. *In vitro* tests, carried out as previously described,[1] show that as little as 25 μg per cc will inactivate a significant amount of virus. Two mg of fraction 6 per egg inhibit multiplication when given as long as 2 hours before or 2 hours after inoculation of virus. Injection of 10 mg into the yolk sac 2 hours before inoculation of virus into the allantoic sac does not inhibit multiplication.

Discussion. Obviously, attempts at purification have been crude, and probably the fractions prepared are mixtures of uncertain composition. Some fractions should contain tannic acid as an appreciable amount is present in tea.[2] Furthermore, as tannic acid is said to be soluble in alcohol, one would expect any present to be concentrated in the alcohol soluble fractions (No. 2 and 7). If inhibitory activity were due to tannic acid the alcohol insoluble fraction (No. 3) and that precipitated at a concentration of 81 to 88% alcohol (No. 6) should show relatively little activity. However, the reverse is true, these fractions being among the most active ones obtained. These differences in solubility may be more apparent than real, depending upon other variables such as pH, which have not been investigated fully. Common tests for tannic acid, such as those employing ferric salts and soluble protein, which are notoriously non-specific, have failed to differentiate clearly the various fractions from each other and from tannic acid. The difficulty of deciding whether the active principle is or is not tannic acid or one of the tannins is further emphasized by the fact that the different natural tannins exhibit differences in their chemical reactions and behavior with solvents.[2] Furthermore, even the "purified" or "crystalline" preparations of tannic acid are mixtures, as it is well recognized that they are contaminated with various impurities.[3] Although the effect of fraction 6 on influenza virus is similar to that of tannic acid, the most significant difference may be a biological one, namely, that the former, although only one-half as active as tannic acid, is some four times less toxic.

Summary. Extracts of tea have yielded an unidentified fraction, similar to but less toxic than tannic acid USP, which inhibits the multiplication of influenza virus in embryonated eggs.

It is a pleasure to acknowledge the technical assistance of Miss Ann Holloway.

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[2] Nierenstein, M., Tannins, in Allen's Commercial Organic Analysis, Vol. V, 5th edition, P. Blakiston's Son and Co., Philadelphia, 1927.

[3] Nierenstein, M., The Natural Organic Tannins, J. and A. Churchill, Ltd., London, 1934.

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17090. Effect of *l*-Epinephrine and *l*-Arterenol on Egg White Edema in the Rat.*

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A number of reports[1,2,11] have described an extensive edema resulting from the intraperitoneal or intravenous administration of egg white in the rat. The cause of the edema is unknown. There is no evidence that it results from a foreign protein reaction as part of a protein hypersensitivity peculiar to the species. The edema may be of a type similar to *p*-phenylenediamine edema which has a peculiar cephalic distribution in cats, rabbits[3-9] and man[10] and which does not depend on previous exposure to the causative agent.

Selye[1] and Leger and Masson[11] observed that "alarming stimuli" inhibit the reaction and the latter workers also described a protective effect of antihistamines in large doses as did Brown and Werner[12] a fact which we have confirmed.† We have noticed that the edema is prevented by much smaller amounts of phenylethylamine pressor drugs and the object of the present communication is to record this observation.

Experimental. Freshly filtered or lyophilized and redissolved raw eggwhite in doses of 0.5 ml was injected intraperitoneally into albino rats of the Slonaker strain, of either sex, and of body weight ranges of 100 to 400 g. The incidence and degree of the edema of the face and extremities were estimated three hours afterward. The incidence was expressed as percentage and the degree by the 0 to 4 + method, averaging the reactions of the face, forefeet and hind feet.

The drugs were injected subcutaneously at the same time as the eggwhite. The drugs reported here included *l*-epinephrine-HCl (Parke, Davis) and *l*-arterenol-HCl (Sterling-Winthrop).

In supplementary experiments to determine the effect of injection route and solvents, *l*-epinephrine-HCl in saline was injected intramuscularly 30 minutes after the eggwhite, and in another experiment subcutaneously in oil.[13] In both cases a comparison was made with the effect of epinephrine injected subcutaneously at the same time as the eggwhite.

Results. The results are summarized in Table I which includes 403 rats.

The results show that under the experimental conditions used, the minimal preventative dose of *l*-epinephrine-HCl is about 100 micrograms/kg when injected subcutaneously at the same time as the eggwhite. When injected intramuscularly, 30 minutes after eggwhite, a slightly greater protection occurred; and when injected subcutaneously in oil, the protective dose was about half this. The minimal preventive dose of *l*-arterenol-HCl was about 2500 μg/kg, thus, this substance is about 1/25 as active as *l*-epinephrine-HCl. Isuprel-HCl [*l*-(3′,4′-dihydroxyphenyl)-2-isopropylaminoethanol-HCl], a "sympathin-I-mimetic" depressor amine, was inactive up to doses of 25 mg/kg which were toxic.

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* Supported by a grant from the Life Insurance Medical Research Fund.

[1] Selye, H., *Endocrinol.*, 1937, **21**, 169.

[2] Leger, J., Masson, G., and Prado, J. L., Proc. Soc. Exp. Biol. and Med., 1947, **64**, 366.

[3] Hanzlik, P. J., *J. Ind. Hyg.*, 1923, **4**, 386.

[4] Gibbs, O. S., *J. Pharm. Exp. Therap.*, 1923, **20**, 221.

[5] Tainter, M. L., and Hanzlik, P. J., *ibid.*, 1924, **24**, 179.

[6] Tainter, M. L., *ibid.*, 1926, **27**, 201.

[7] Tainter, M. L., *ibid.*, 1928, **33**, 129.

[8] Gibbs, O. S., *ibid.*, 1931, **42**, 65.

[9] Cohen, M. B., *ibid.*, 1933, **48**, 235.

[10] Malosetti, H., Fernandez, G. J., and Migliaro, E., *Arch. Uruguayos Med. Cir. y Espec.*, 1947, **31**, 25.

[11] Leger, J., and Masson, G., *Ann. Allergy*, 1948, **6**, 131.

[12] Brown, B. B., and Werner, H. W., *J. Lab. Clin. Med.*, 1948, **33**, 325.

† Pyribenzamine and phenergan [(dimethyl-amino-2-methyl-1-ethyl)-N-dibenzo-*p*-thiazine] were tested. The latter was over twice as effective as the former. 10 mg/kg administered subcutaneously at the same time as the eggwhite injection nearly completely inhibiting the edema, while for pyribenzamine, 25 mg/kg were required.

[13] Keeney, E. L., *Bull. Johns Hopkins Hosp.*, 1938, **62**, 227.

TABLE I.
Effect of *l*-Epinephrine and *l*-Arterenol on Eggwhite Edema in Rats.

Drug used	Dose, µg/kg	No. indiv. exp.	No. rats used	Avg % incidence of edema	Avg degree of edema in affected rats
None	0	16	94	90%	2.5+
Epinephrine subcutaneous	5	2	12	85	4
,, ,,	25-30	5	49	95	3
,, ,,	35-40	5	32	90	3
,, ,,	50-55	8	64	95	3.5
,, ,,	60-75	3	21	65	3.5
,, ,,	100	2	11	10	trace
,, ,,	140-150	2	10	40	,,
,, ,,	200	1	5	20	,,
,, ,,	275	1	5	0	0
,, ,,	550	1	5	0	0
Epinephrine intramuscular	25	1	8	100	3.0
,, ,,	50	2	14	50	1.5
,, ,,	75	1	6	35	2.5
,, ,,	100	2	14	10	trace
,, ,,	200	1	8	0	0
Epinephrine in oil, subcut.	40	1	5	0	0
Arterenol subcutaneous	100	2	10	90	2.5
,, ,,	200	1	5	60	2.5
,, ,,	400	1	5	80	2.5
,, ,,	500	1	5	80	2.5
,, ,,	1000	1	5	80	1.0
,, ,,	2500	1	5	20	trace
,, ,,	3000	1	5	0	0

Discussion. Gibbs, Hanzlik and Tainter[4-7] found that sympathetic nerve stimulation, drugs which cause release of epinephrine from the adrenal medulla, and vasoconstrictor drugs all inhibit *p*-phenylenediamine edema. Eggwhite edema is similar in this respect. Selye's[1] and Leger and Masson's[11] observations of a protective effect of "alarming stimuli" might be interpreted as a result of release of epinephrine from the adrenal medulla. Experiments designed to elucidate this point are in progress.

In the course of this work, it was observed that intravenous Evans' blue dye was extravasated into the edematous areas of the eggwhite response, notably the feet, ears, nose, lips and tongue. This is similar to *p*-phenylenediamine injury.[5] In both types of edema, *l*-epinephrine is more inhibitory than other pressor substances studied thus far. The present study would indicate therefore that *l*-epinephrine is a much more powerful arteriolar vasoconstrictor (depressor of capillary blood pressure) than *l*-arterenol. In spite of this, the latter has been reported to be 1 to 5 times more active than *l*-epinephrine in raising the arterial blood pressure[14-16] and has been thought to be the augmentor hormone of the sympathetic nervous system.

The antihistamines tested, pyribenzamine and phenergan, were 1/100 and 1/250 as active, respectively, as *l*-epinephrine, in preventing the edema.

Summary. l-epinephrine in a subcutaneous dose, 100 µg per kg, inhibits eggwhite edema in the rat. *l*-arterenol is about 1/25 as effective. Isuprel is inactive.

Since submitting the present manuscript, several papers have appeared which have demonstrated the presence of considerable quantities of *l*-arterenol in naturally derived epinephrine preparations, such as the one used in the present study. The use of synthetic *l*-epinephrine, therefore, would have enhanced the difference in activity of *l*-epinephrine and *l*-arterenol reported here.

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[14] Tainter, M. L., Tuller, B. F., and Luduena, F. P., *Science*, 1948, **107**, 39.

[15] Euler, U. S. von, *Acta Physiol. Scand.*, 1948, **16**, 63.

[16] Luduena, F. P., Ananenko, E., Siegmund, O. H., and Miller, L. C., *J. Pharm. Exp. Therap.*, in press.

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17091. Paper Chromatographic Identification of Some N-substituted Amino Acids.*

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Ever since Consden, Gordon and Martin[1] introduced the use of paper chromatography it has been successfully applied to the resolution of a great many biological mixtures. In the work presented here, paper chromatography was applied to the separation of a group of amino acid analogs studied for their tumor growth inhibitory effect on the transplanted sarcoma 37 in strain "A" mice.

The ascending capillary technic of Williams and Kirby[2] was adopted for the study of the separation of N-substituted amino acids in quantities of 25 γ. The paper strips were suspended from paper clips carried by a toothed non-corroding nickel alloy support which was firmly attached to the heavy glass of the jar. This toothed support permitted the simultaneous employment of 6 strips of Whatman No. 1 filter paper (40 × 12 cm). The solvent mixtures employed were phenol-water and collidine-water. After the solvents reached a 20 cm liquid front (L.F.) value which was within 20 hours, the strips were removed and dried in air with the aid of an electric fan. In developing the ninhydrin reaction the papers were kept at 90° for 10 minutes. The dried strips were then examined under an ultraviolet lamp (General Electric bulb BH4). The free amino acids showed up as faint blue spots, whereas the N-substituted amino acids were recognized by the presence of absorbing, non-emitting dark spots on the paper. After the ultraviolet analysis, the paper strips were sprayed with a 0.15% ninhydrin solution in saturated butanol-water.

Both the free amino acids and substituted analogs responded with spot formation on the positions outlined by previous ultraviolet analysis. The N-substituted analogs, however, gave spots far below the intensity of those of their corresponding amino acids. It was found that both color intensity and R_f values could be correlated with the rate of hydrolysis of the substituent and with the character of the steric arrangement. Thus, the size of the isopropyl chain is somewhat smaller than that of the propyl, which expresses itself in the somewhat greater R_f value of the latter. The phenyl N-substituted amino acids, however, disappeared from the paper either by this method of drying or by heat drying. The phenyl derivatives showed definite melting points between 110 and 180° and were quite soluble in organic solvents. Thus, it is very likely that the small amounts used are removed along with the solvent vapors from the paper strips during drying before any spot development can be observed, for, if control paper strips, untreated with solvent mixtures, carrying the phenyl derivatives were treated with ninhydrin solution, spots characteristic of traces of unsubstituted amino acid showed up, just as in the case of the other N-substituted amino acid-analogs.

Table I represents the tabulated R_f values of certain of the N-substituted and of the corresponding free amino acids. It must be emphasized that the spots developed by ninhydrin can not be attributed to the presence of traces of amino acids as impurities, as synthesis of the analogs did not employ amino acids at any stage of the work. Furthermore, the microanalytical values of the compounds agreed closely with the calculated ones. As the table shows, R_f values vary greatly according to the concentration of water in the solvent mixtures. In 65% phenol the N-substituted amino acids move almost as fast as the liquid front. Separation is not sharp and use of this solvent mixture becomes practical only in two dimensional work provided the chromatogram is developed first with 71%

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* This work was supported by a grant from the Cancer Research Grants Branch, U. S. Public Health Service, to Dr. David M. Greenberg.

† U. S. Public Health Special Fellow.

[1] Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224.

[2] Williams, R. J., and Kirby, H., *Science*, 1948, **107**, 481.

TABLE I.
R_f Values.

Compounds	Phenol 65%	Phenol 80%	Collidin 71%	Collidin 63%
DL-valine	0.73	0.69	0.25	0.43
Ethyl-N-DL-valine	0.94		0.41	
Isopropyl-N-DL-valine	0.97		0.43	
Propyl-N-DL-valine	0.99		0.44	
Phenyl-N-DL-valine*				
DL-leucine	0.81	0.74	0.37	0.55
Ethyl-N-DL-leucine	0.92		0.46	
Isopropyl-N-DL-leucine	0.96		0.56	
Propyl-N-DL-leucine	0.99		0.58	
Phenyl-N-DL-leucine*				
DL-phenylalanine	0.83	0.76	0.39	0.58
Ethyl-N-DL-phenylalanine	0.94		0.53	
Isopropyl-N-DL-phenylalanine	0.97		0.59	
Propyl-N-DL-phenylalanine	0.99		0.61	
Phenyl-N-DL-phenylalanine*				

* The phenyl derivatives evaporated from the paper upon drying (see text).

collidine and with phenol afterwards. It is important, as other authors have also suggested, that one run a control chromatogram of a known amino acid, to establish the necessary correction for the R_f values of the solvent mixtures used.

Of further interest is the observed degree of ninhydrin color sensitivity of the N-substituted amino acids. Recently Dent[3] reported that sarcosine reacts with an intense color formation. Plattner and Nager[4] have observed positive ninhydrin reactions with methyl-N-DL-valine and methyl-N-L-leucine. It was found that these compounds boiled with ninhydrin did not produce any aldehydes or ketones, because they do not have two hydrogen atoms on the nitrogen. The appearance of the ninhydrin reaction was positive irrespective of whether the paper was exposed to ultraviolet light or not. The positive ninhydrin color may be interpreted as being the result of a certain degree of hydrolysis that takes place under the effects of the solvents and heating that are used to develop the chromatograms. The intensity of the reaction decreases with increase of chain length in the homologous series of alkyl substitutions. The R_f values, on the other hand, increase with the length of the substituents, in as much as the n-alkyl group exhibits a somewhat faster rate of movement than the corresponding branched chain isomer.

Summary. N-substituted amino acid can be separated by paper chromatographic method. Also, N-substitution leads to the extinction of fluorescence of amino acids.

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[4] Plattner, P. A., and Nager, U., *Helv. Chim. Acta*, 1948, **31**, 2203.

17092. Importance of Potassium and Magnesium in Nutrition of the Guinea Pig.*

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Several extensive studies have been made to devise a purified ration which will give growth in guinea pigs equivalent to that obtained with commercial rations (about 7 g/day). Booth *et al.*[1] recently found that powdered gum arabic, when added to a purified synthetic ration at a level of 15%, increased the daily growth from 1.8 to 5.1 g. When in addition to gum arabic the ash from alfalfa leaf meal was added to the ration at a level equivalent to 25% of alfalfa, nearly "normal" growth was obtained. The particular constituents of alfalfa ash responsible for the activity observed were not identified. In this paper we report experiments which were conducted to determine the active components of alfalfa ash.

Experimental. The experimental procedure was similar to that of Booth *et al.*[1] The synthetic basal ration consisted of: sucrose 60.9%, casein (vitamin-free) 30%, salts IV[2] 4%, fortified soybean oil 4%, sucrose mixture containing B vitamins 0.8%, and choline 0.3%. The fortified soybean oil supplied 1.2 mg of β-carotene, 12 mg of α-tocopherol, 8 μg of calciferol and 0.2 mg of menadione per 100 grams of ration. The vitamin B mixture supplied 1 mg of thiamine hydrochloride, 1.4 mg riboflavin, 1 mg pyridoxine, 3 mg calcium pantothenate, 200 mg inositol, 10 mg niacin, 10 mg p-aminobenzoic acid, 0.04 mg biotin and 0.3 mg folic acid per 100 grams of ration. Vitamin C dissolved in sucrose solution was fed with individual pipettes at a level of about 25 mg every other day. Gum arabic was used at a level of 15%. The gum and the different inorganic compounds tested were added to the ration in place of an equal amount of sucrose.

Results. Since it seemed possible that the beneficial effect of alfalfa ash observed by Booth *et al.* was due to its alkalinity, certain alkaline compounds were used in place of the ash. It was noticed in preliminary experiments that the addition of potassium acetate or magnesium oxide to the basal ration containing gum arabic distinctly improved the growth. Very good growth was obtained with mixtures of these two minerals. The results of an experiment in which different levels of potassium acetate and magnesium oxide were fed are given in Table I. The best growth was obtained when 2.5% potassium acetate and 0.5% magnesium oxide, *i.e.*, 1.0% K and 0.3% Mg, were added to the ration. The amounts of K and Mg in the basal ration supplied by salts IV are 0.56% and 0.04%, respectively.

Table II shows the results of experiments in which other compounds of K and Mg were compared with potassium acetate and magnesium oxide. The amount of potassium acetate used in the rations given in Table II was 2.5% and an equivalent amount of potassium was given as potassium bicarbonate and phosphate. Magnesium oxide was used at a level of 0.5% and an equivalent amount of magnesium was included in the other magnesium compounds. The data show that the growth of the animals receiving potassium acetate and magnesium oxide but no gum arabic was rather poor. However, growth equal to that of animals on the stock ration was obtained, when these minerals were fed in the presence of gum arabic. An experi-

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by funds granted by the National Dairy Council, Chicago, on behalf of the American Dairy Association. We are indebted to Merck and Co., Inc., Rahway, N. J., for supplying the synthetic B vitamins and α-tocopherol and to the Winthrop Chemical Co., Inc., New York, for crystalline vitamin D_2.

† Rockefeller Foundation Fellow.

[1] Booth, A. N., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, 1949, **37**, 263.

[2] Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

TABLE I.
Effect of Different Amounts of Potassium Acetate and Magnesium Oxide on Growth of Guinea Pigs Receiving a Purified Basal Ration Plus 15% Gum Arabic.

Potassium acetate, %	Magnesium oxide, %	No. of animals	Avg gain in wt during 6 wks, g/day
None	None	4	5.2
2.5	0.25	4	5.3
2.5	0.5	5	7.2
2.5	1.0	4	5.1
5.0	1.0	4	6.2
1.25	0.5	3	6.2

TABLE II.
Growth of Guinea Pigs on Different Rations.

Ration	No. of animals	Avg gain in wt during 6 wks, g/day
Basal + gum arabic	4	5.2
Basal + potassium acetate + magnesium oxide	4	2.6
Basal + gum arabic + potassium acetate + magnesium oxide	13	6.4
Basal + gum arabic + potassium acetate + magnesium carbonate	4	6.2
Basal + gum arabic + potassium acetate + magnesium acetate	4	7.1
Basal + gum arabic + potassium acetate + magnesium sulfate	4	5.4
Basal + gum arabic + potassium bicarbonate + magnesium oxide	4	5.2
Basal + gum arabic + potassium dihydrogen phosphate + magnesium oxide	3	5.5
Rockland stock diet	9	6.6

ment lasting 11 weeks showed that the "normal" growth on the ration containing potassium acetate and magnesium oxide continued at least this length of time. Of the other salts tested magnesium acetate showed a very good growth response. Further experiments with larger numbers of animals are required, however, to show the comparative values of different salts.

Since potassium acetate and magnesium oxide were not active in the absence of the gum arabic, it appears that they may produce conditions in the tract which allow the presence of an intestinal flora more beneficial to the host. The assumption that they may raise the pH in the cecum is probably not correct because it was found that the pH of the cecum was the same (average 6.9) irrespective of whether or not the diet contained added K and Mg.

Summary. A normal rate of growth for guinea pigs, comparable with the growth on commercial rations, was obtained when potassium acetate and magnesium oxide were added to a purified basal ration containing all of the known nutrients (except vitamin B_{12}) and 15% gum arabic. The best results were obtained with 2.5% potassium acetate and 0.5% magnesium oxide.

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17093 P. Infection of Mammalian Erythrocytes by the Avian Malaria Parasite, *Plasmodium lophurae*.*

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Reports concerning attempted infection of mammals with avian malaria parasites are limited, although recently Beckman obtained negative results following introduction of *Plasmodium cathemerium* parasites into man[1] and the guinea pig.[2] In a series of experiments unrelated to the immediate problem, the author noted that guinea pig erythrocytes injected into normal 10 day chick embryos survived for a period of 4 days. As a result of this finding, it was decided to investigate the possibility of securing infection of mammalian cells in chick embryos infected with *P. lophurae,* a parasite to which chick embryos have been found to be highly susceptible.[3]

White leghorn embryos of 10 days' incubation were given intravenous injections of 3 × 10^7 parasites, representing the 24th passage of *P. lophurae* in embryos. Two tenths cc each of twice washed erythrocytes of mouse, rat, rabbit, dog, sheep, and man were injected intravenously into embryos on the 2nd day after parasite introduction, at which time the infections averaged about 1100 per 10,000 avian erythrocytes. Blood films were usually made twice daily thereafter, although in certain embryos 4 or 5 smears were made in a 24 hour period. Giemsa's stain was used. Only cells agreeing in size, morphology, and staining reactions with the mammalian cell introduced were considered in determination of infection, since during the later stages of embryo infection artifacts arose which might have led to false identification.

Dog and rabbit erythrocytes were not well adapted to survival in chick embryos, the former killing the embryo in a period of 12 hours, the latter disintegrating rapidly. Sheep and rat red blood cells were well preserved even after 3 days but were uninfected. Guinea pig cells, although surviving better than those of the rabbit, disintegrated to the point that they were difficult to separate from abnormal avian erythrocytes. Therefore, although many infected cells suggesting those of the guinea pig were seen, the results were considered negative. Human erythrocytes retained their characteristic morphology throughout the period studied. Two infected cells were found, both with early trophozoites of *P. lophurae.* Infections were so low, however, that this type of cell was regarded as unsuitable for further study.

Erythrocytes of Swiss mice maintained their morphology and were readily parasitized. The proportion of mammalian to avian erythrocytes was usually 1:2. Infected mouse cells were found as early as 12 hours after introduction, although the percentage of infected erythrocytes remained low never exceeding 10 per 10,000. Very few doubly infected cells were observed. Early trophozoites were similar to corresponding stages in the avian erythrocytes. More mature trophozoites frequently elongated into the band forms characteristic of *P. malariae* but, like this parasite, they were sometimes seen as spherical masses in the center of the host cell (Fig. 1a). Pigment granules were small, golden-brown spheres. The cytoplasm was homogenous and stained similarly to *P. lophurae* parasites in chicken erythrocytes. Nuclear structures of trophozoites were identical with *P. lophurae* in chicken red blood cells. After two nuclear divisions parasites occupied a large proportion of the host cell and in later stages caused the cell to enlarge. Pre-segmenting forms with 7-10 nuclei were seen after 32 hours, and fully segmented parasites were seen 36 hours following introduction of erythrocytes (Fig. 1b) at

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* The author wishes to express his gratitude to Dr. W. Trager for constructive criticism and generous assistance throughout this work.

[1] Beckman, H., Proc. Soc. Exp. Biol. and Med., 1947, **66**, 401.

[2] Beckman, H., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 172.

[3] McGhee, R. B., *J. Parasitol.*, 1949, in press.

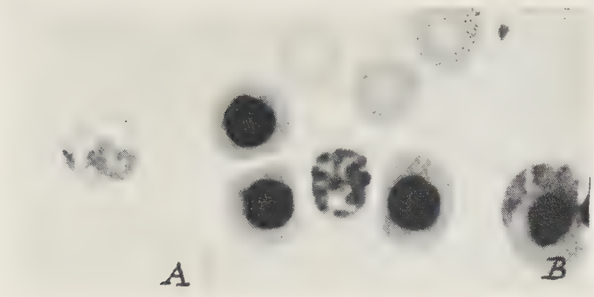

FIG. 1a. A medium trophozoite of *P. lophurae* in a mature mouse erythrocyte. ×1770.

FIG. 1b. A segmenting parasite of *P. lophurae* in a slightly enlarged mouse erythrocyte, from a smear made 36 hours following introduction of mammalian cells. ×1600.

which times avian cell infections averaged about 8000 per 10,000 red blood cells. Five to 11 merozoites, smaller than those in avian erythrocytes, were produced, generally in a rosette form. No gametocytes were found.

Cultures prepared according to the formula of Trager[4] using whole mouse blood in conjunction with infected chick embryo blood indicated a certain amount of invasion of mouse erythrocytes *in vitro*.

In view of the high degree of host specificity of avian malarias, it is surprising that cells of hosts so distantly related as to be placed in another phylum are invaded. Parasitism of mouse cells might, therefore, demonstrate that in certain cases the resistant principle lies in the serum constituents, rather than in the erythrocyte. *P. lophurae* is transmitted readily by *Anopheles quadrimaculatus* and this fact, along with the successful infection of mouse erythrocytes, might indicate a closer relationship between *P. lophurae* and mammalian malaria parasites than was previously supposed.

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[4] Trager, W., *J. Parasitol.*, 1947, **33**, 345.

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17094. A Falling Sphere Method for Studying Clotting in Systems of Purified Blood Components.

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(Introduced by Francis O. Schmitt.)

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Although the conversion by thrombin of fibrinogen to fibrin is generally used as an indicator of reactions occurring earlier in the process of blood coagulation, methods for the study of this conversion, with very few exceptions, measure only a single end-point in fibrin formation.[1] It is apparent that a method which measures quantitative changes in the coagulation system up to some definite end-point might be of value in following the kinetics of the system. An involved technic has been reported[2] for measuring early alterations in clotting systems by recording changes in intensity of transmitted light as coagulation proceeds. Methods based upon changes in viscosity of a clotting solution have

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[1] Quick, A. J., The Hemorrhagic Diseases and the Physiology of Hemostasis, Charles C. Thomas, Springfield, 1942.

[2] Nygaard, K. K., Hemorrhagic Disease. Photoelectric Study of Blood Coagulation, C. V. Mosby Company, St. Louis, 1941.

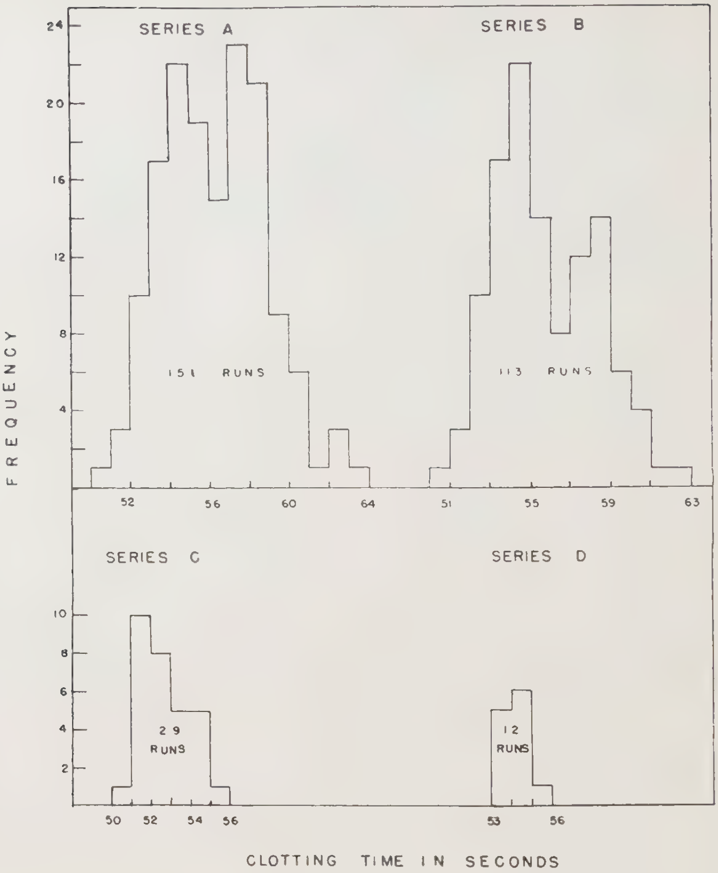

FIG. 1.

Frequency distribution curves of coagulation times, as measured by the falling sphere method, of many different experiments on the thrombin-fibrinogen system. For the experimental conditions of each series of runs, see text.

been described but not in quantitative terms.[3] The purpose of this study was to develop and standardize a quantitative technic for measuring coagulation employing purified thrombin and fibrinogen. This method is based upon the measurement of gelation which occurs in a system during the conversion of fibrinogen to fibrin and utilizes Stoke's law for a falling sphere in the measurement of viscosity.

Materials and methods. Bovine thrombin (Upjohn) was dissolved in 0.85% NaCl solution in a concentration of 20 units per milliliter immediately before use. Fraction I of bovine plasma (Armour) was employed as fibrinogen in a concentration of 0.2% in 0.85% NaCl solution; the fresh solution was filtered through analytical filter paper before using. After adjusting all preparations to 25 ± 0.3°C by immersing in a thermostatically controlled water bath, three-tenths milliliter of fresh thrombin solution was added to fifteen milliliters of fibrinogen solution at zero time and rapidly mixed by a rotary movement of the beaker. A pyrex glass tube eight millimeters in diameter, forty centimeters long and stoppered at one end was filled to the brim with the mixture and placed vertically in the water bath with about 3 cm of the upper end of the tube above the surface. As the solution became opaque a plexiglass bead* (moistened with saline) was inserted in the upper end of the tube and allowed to fall to an abrupt stop. The elapsed time from mixing of solutions to this sharp end-point was taken as the clotting time. Fibrin or air bubbles clinging to the bead disturbed its fall and rendered the end-point uncertain. The pH was 6.2 in both clotted and unclotted preparations.

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[3] Hedenius, P., *Acta med. Scand.*, 1936, **88**, 440.

Results. Series A (Fig. 1) shows the statistical variation in 151 runs before any standardization of the technic as described above was made. The arithmetical mean is 56.2 seconds and the mode is the same indicating a normal distribution curve. The standard deviation is 2.87%.

Series B shows the scatter of experimental data obtained in 113 runs after standardization of the technic but employing fibrinogen solutions varying in age from only a few minutes to several hours. The arithmetical mean is 55.7 seconds, the mode is 55.2 seconds and the standard deviation is 2.5%.

Series C includes data of 29 runs in which the variation caused by deterioration of the fibrinogen solution was reduced by using a freshly prepared solution every 10 runs (the solution was always less than one and a half hours old when used). The arithmetical mean is 53.5 seconds, the mode is 55.3 seconds and the standard deviation is only 0.98%.

Series D shows the accuracy obtained when the errors of weighing, diluting, etc. of different samples are minimized by using the same solutions of fibrinogen and thrombin throughout 12 runs. The runs were made in less than one and a half hours in order to minimize the effect of aging on the thrombin and the fibrinogen. The arithmetical mean is 54.1 seconds, the mode is 54.1 seconds and the standard deviation is 0.49%.

Deterioration of fibrinogen and thrombin in solutions standing at 25°C was manifested by a gradual prolongation of the clotting time. Fig. 2 illustrates the rapid increase in clotting time of a fibrinogen solution as demonstrated by coagulating aliquots of it at successive intervals over a period of six hours using a fresh thrombin solution. The activity of thrombin solutions also changed on standing, but at a very low rate as illustrated in Fig. 3.

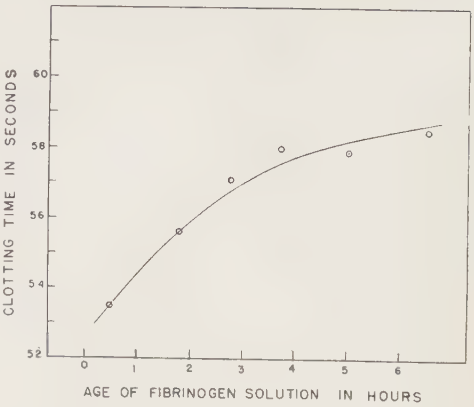

FIG. 2.

The clotting time is shown to increase with the age of a fibrinogen solution allowed to stand over 6 hours at 25°C.

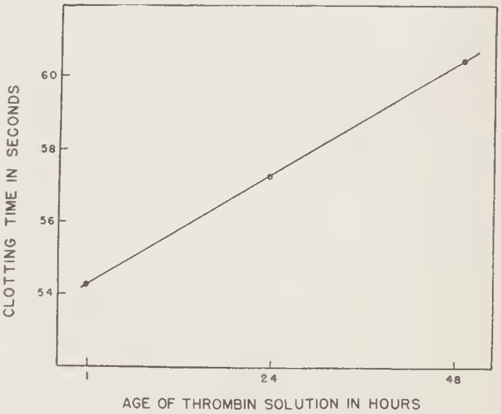

FIG. 3.

Clotting time is shown to increase with the age of a thrombin solution allowed to stand over 48 hours at 4°C.

By inserting successive beads at various intervals before gelation was complete and timing the rate of fall of the beads over a measured length of tube, the data for the kinetics curve of Fig. 4 were obtained. The rate of fall of the bead is plotted as a function of the duration of the action of the thrombin upon the fibrinogen. There was no apparent change in the viscosity of the mixture

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* Spherical beads were made of plexiglass (lucite), 3.25 ± 0.1 mm in diameter and weighing 21.6 mg. They were standardized for uniformity by timing their fall over a definite distance in a tube filled with fibrinogen solution.

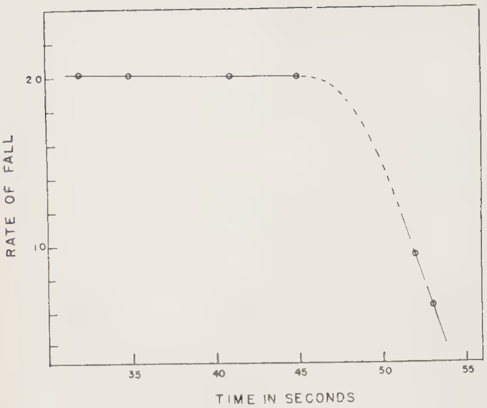

FIG. 4.

The rate of fall of plexiglass beads, expressed in centimeters per second, is plotted as a function of the time elapsing between mixing fibrinogen with thrombin and inserting a bead into the solution. Beads were allowed to fall into the solution 30 seconds after mixing the thrombin and fibrinogen and at 5-10 second intervals after that until gelation had occurred.

for approximately the first 46 seconds of the reaction. Then followed a rapid increase in viscosity leading to gelation and a cessation of falling of the bead. The reproducible clotting times that we have been able to obtain in the statistical study find a ready explanation in the abrupt change in the curve associated with coagulation.

Using 0.3, 0.4, 0.5 and 0.6 ml of thrombin solution (concentration 10 units per ml) average clotting times of 106, 84, 69, and 60 seconds respectively were obtained. (At each dilution the range of readings was ± 3% of the average clotting time). These results indicate a roughly linear relationship between thrombin concentration and rate of clotting.

Three different commercial lots of fibrinogen gave different absolute clotting times under standard conditions owing partially at least to variation in solubility and "percent of clottable protein" in the plasma fraction I. However, different samples of the same lot gave consistent results. The thrombin showed much less variation of activity between lots.

Summary. 1. To study the coagulation of the fibrinogen-thrombin system, a simple rapid method has been developed which measures the time required for the developing coagulum to stop the fall of a plastic bead in a glass tube containing the fibrinogen-thrombin mixture.

2. The "clotting time" thus obtained, increases somewhat with the age of the thrombin and fibrinogen solutions.

3. Kinetic studies of the coagulation process indicate that there is no change in viscosity for a considerable time after the mixing of thrombin and fibrinogen, but that this phase is followed by an abrupt increase in viscosity signifying coagulation of the system.

4. The rate of clotting varies with thrombin concentration in a roughly linear fashion.

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17095. A New Serological Test (Inhibition Test) for Human Serum Globulin.

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Moreschi[1] described a special serological technic by which it is possible to demonstrate the presence in immune sera of antibodies for red cells or bacteria in higher titers than by the classic agglutination technic. His technic consisted in first titrating the antiserum against the test blood or bacterial suspension in saline media and subsequently adding a precipitin serum against the serum of the animal providing the antiserum for the erythrocytes or bacteria. The following precautions were necessary in order that this test might work: (1) The precipitin serum had to be absorbed with the washed erythrocytes or bacteria used as antigen, in order to remove any natural hemagglutinins or bacterial agglutinins which might be present in the serum. (2) After the first stage of the reaction was completed and before the precipitin serum was added, the sensitized cells were washed several times with large volumes of saline in order to remove the supernatant serum proteins which could inhibit the reaction. Moreschi thought that his test served to accentuate the reaction of immune *agglutinins*. Actually, we now know, principally as result of the recent work on Rh antisera, that the test is specific for so-called *univalent antibodies* or *glutinins*.

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[1] Moreschi, C., *Centralbl. f. Bakteriol.*, 1908, **46**, 49; *ibid.*, 1908, **46**, 456.

Following the demonstration by one of us[2,3] that Rh-negative individuals sensitized to the Rh factor may produce two sorts of Rh antibodies, namely, Rh agglutinins (bivalent antibodies) and Rh blocking antibodies or agglutinins (univalent antibodies), Coombs *et al.*[4] suggested the application of the anti-globulin test as a means of detecting univalent Rh antibodies. The purpose of the present communication is to describe a modification of the anti-globulin test by which it is possible to identify human serum globulin specifically and estimate its concentration.

Materials and method. The principle of the absorbed, diluted anti-human-globulin is as follows. The solution to be tested for the presence of serum globulin is mixed with the absorbed, diluted anti-human-globulin serum and the mixture allowed to react in a water bath at 37°C for one hour. Then a suspension of Rh-positive cells, coated with Rh_o univalent antibody, is added and the mixture allowed to stand for a second period of incubation, after which the reaction is read. Failure of clumping to occur indicates the presence of human serum globulin in the material being tested; the occurrence of clumping indicates the absence of human serum globulin.

The sensitized Rh-positive cells used in the test were prepared as follows. A fresh, 2% suspension of Rh_o-positive red cells in saline was prepared and washed once with saline solution. This suspension was mixed with an equal volume of an Rh_o antiserum containing univalent antibodies with a titer of approximately 100 units by the albumin-plasma method.[5] This mixture was allowed to react in the incubator at 37°C for 45 minutes. The tube was then centrifuged at moderate speed, the supernatant fluid discarded, and the sedimented cells washed four times with large volumes of saline solution. The sensitized cells were then resuspended in sufficient saline to make a concentration of approximately 2%.

The antiglobulin serum used in these experiments had been prepared in the usual way and was kindly provided by Drs. Peter Vogel and Richard Rosenfield.

In the experiments to be described the following materials were tested: normal human adult and umbilical cord serum; human spinal fluid, saliva, semen and urine; and normal animal serum from horse, ox, rabbit, and rhesus monkey. The tests were carried out quantitatively by preparing progressively doubled dilutions in saline solution of the material to be tested and mixing a drop of each dilution, in a corresponding series of small test tubes, with a drop of the anti-human globulin serum. After 45 minutes incubation at 37°C, a drop of the suspension of Rh-positive, sensitized cells was added to each tube; the tubes were shaken and reincubated for 60 to 90 minutes. The tubes are then gently shaken and the reactions read with the naked eye and under the low power of the microscope.

Results. Table I contains a selection of the results obtained in the various experiments. The tests shown in the table were not all done on the same day but are combined for purpose of comparison.

With the technic used, normal adult human serum completely inhibits the agglutination reaction of the anti-human-globulin serum in dilutions as high as 1:500, on the average. It is necessary to emphasize at the onset that the method shares the limitations of all other serological titrations, namely, a lack of precise reproducibility. In tests made on different days or even on the same day, variations in titer as great as two serum dilutions

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[2] Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 173.

[3] Wiener, A. S., *J. Lab. and Clin. Med.*, 1945, **30**, 662.

[4] Coombs, R. R. A., Mourant, A. E., and Race, R. R., *Brit. J. Exp. Path.*, 1945, **26**, 255.

[5] Wiener, A. S., and Hurst, J. G., *Exp. Med. and Surg.*, 1947, **5**, 285.

TABLE I.

Results of Inhibition Tests, Using Rabbit Anti-Human-Globulin Serum and Rh-Positive Erythrocytes Coated with Anti-Rh$_0$ Glutinin.

Material tested	Dilution of material tested													
	Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:5096	1:10,192
Normal human serum (adult)	—	—	—	—	—	—	—	—	—	—	±	+±	++	++
” ” ” (umbilical cord)	—	—	—	—	—	—	—	—	—	—	tr.	+±	++	++
10% purified human globulin	—	—	—	—	—	—	—	—	—	—	—	—	—	±
25% ” ” albumin	++	++	++	++	++	++								
Normal human spinal fluid	—	—	±	++	++	++	++	++						
Spinal fluid (4+ Wassermann)	—	—	—	—	±	++	++	++						
Normal human urine	+	+±												
” ” saliva	—	—	+	±	+±	++	++	++						
” ” semen	+±	+±												
Normal horse serum	++	++	++	++	++	++								
” ox ”	++	++	++	++	++	++								
” rabbit ”	++	++	++	++	++	++								
” rhesus ”	+	+	+	+	+±	+	±	+	+	+	+	+±	++	++

were not unusual, so that only a tolerable degree of accuracy was possible by averaging the results of several titrations. In addition the end points of the inhibition titrations were often difficult to determine due to the irregularity of the degree of clumping in successive tubes in many of the experiments.

In an attempt to determine which serum fraction is responsible for the inhibition of the anti-globulin serum, titrations were carried out with purified human serum albumin and purified human serum globulin. The serum albumin used was a 25 percent salt-poor solution purchased from Cutter Laboratories. The serum globulin was a 10 percent solution of Squibb's Red Cross Gamma Globulin, kindly provided by Dr. P. Vogel. As shown in the table, the serum albumin failed to inhibit the anti-globulin serum, while the serum globulin inhibited the serum strongly, in accordance with expectation. These results therefore provide additional proof that the antibody coating the sensitized red cells consists of serum globulin. It is to be noted that the inhibition titer of the purified human globulin is higher than that of unprocessed serum, in proportion to the concentration of globulin.

A few tests were performed with human umbilical cord serum, which gave titers approximately equal to those of normal adult serum. Even assuming that the fetus *in utero* is incapable of producing serum globulin, this finding was to be expected from the fact that univalent maternal antibodies readily traverse the placenta into the fetal circulation until the titers in the maternal and fetal circulation are equal.[6,7]

The normal human spinal fluid inhibited the anti-globulin serum but only up to the dilutions of approximately 1:2 or 1:4. Since normal serum contains approximately 7 g of protein per 100 cc while normal spinal fluid contains only about 30 mg per 100 cc, the inhibition titer of approximately 3 units obtained for spinal fluid corresponds satisfactorily to the expectation, on the basis of an inhibition titer of approximately 500 units for

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[6] Wiener, A. S., and Sonn, E. B., *J. Lab. and Clin. Med.*, 1946, **31**, 1016.

[7] Wiener, A. S., *Ann. Allergy*, 1948, **6**, 293.

blood serum. With abnormal spinal fluid such as those obtained from syphilitic patients, higher inhibition titers were obtained, as to be expected. Saliva gave definite inhibition though only in low titer, in conformity with the known presence in this secretion of small amounts of serum globulin. Only doubtful reactions were obtained with normal human semen and urine.

Experiments were also conducted to determine the ability of animal sera to inhibit the anti-human-globulin serum. As shown in the table, serum from horse, ox, and rabbit had no apparent inhibiting action. Rhesus serum weakened the clumping, even when highly diluted, but did not inhibit the reaction completely. This indicates the presence in rhesus serum of a serum globulin chemically related to, though qualitatively different from the globulins in human serum.

Summary and conclusions. 1. A new serological method of demonstrating human serum globulin has been described, based on its ability to inhibit the agglutinating action of anti-human-globulin serum for Rh-positive human red cells coated with Rh_o univalent antibody.

2. Using this technic, further evidence was obtained that the Rh antibody coating sensitized Rh-positive cells is a serum globulin.

3. With the new technic it is possible to demonstrate the presence in normal spinal fluid, and in saliva of small amounts of serum globulin, in proportion to the known concentration in these fluids. Normal human semen and urine failed to inhibit the antiglobulin serum.

4. Umbilical cord serum inhibited anti-globulin serum to the same titer as adult serum.

5. Serum from horse, ox and rabbit did not inhibit the anti-human-globulin serum, while rhesus monkey serum merely weakened its reactions.

6. The new technic may find application not only in clinical medicine, but also in forensic work for the examination of blood stains, and in studies on biochemical evolution.

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17096. Relationship of Catalase Activity to Virulence in *Pasteurella pestis*.

MORRIS ROCKENMACHER. (Introduced by A. P. Krueger.)

From Office of Naval Research Task V, Department of Bacteriology, and U. S. Naval Medical Research Unit No. 1, University of California, Berkeley, Calif.

Huddleson[1] and Merz[2] observed that virulent *Brucella* organisms showed greater catalase activity than avirulent strains. This investigation was undertaken to determine whether a similar relationship exists for *Pasteurella pestis*. It was felt that such findings might be applied to the development of a rapid, inexpensive, *in vitro* method for virulence titration. Furthermore, such a relationship would indicate a basis for approaching the problem of virulence along the lines suggested by Jawetz and Meyer[3] who state, "It appears probable that the final criterion of virulence will have to rest on a biological and chemical identification of the bacterial enzyme systems which are responsible for the multiplication of the plague bacilli in the animal host."

Procedure. Huddleson's[1] method was modified as follows to measure the catalase activity of virulent and avirulent strains of *P. pestis*. Standard bacterial suspensions were prepared from twice-washed, 24 hour, heart infusion broth cultures suspended in phosphate buffer and adjusted to a turbidity of 100 ± 5 on the scale of the Klett-Summerson photoelectric

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[1] Huddleson, I. F., Univ. Mich. Agri. Exp. Station, Technical Bull. 182, January, 1942.

[2] Merz, P., Uber die Katalasen der Brucellen, Inaugural Dissertation for the Degree of Doctor of Veterinary Medicine, University of Zurich, 1938.

[3] Jawetz, E., and Meyer, K. F., *Am. J. Path.*, 1944, **20**, 457.

TABLE I.
Relationship of Catalase Activity to Virulence in *Pasteurella pestis.*

Pasteurella pestis strains				Virulence		Catalase activity (ml of H_2O_2)			
						Min. after adding bacterial suspension			
No.	Name	Catalase determinations	Viable bacteria ($\times 10^8$/ml)	No. of titrations	LD$_{50}$	5	15	30	60
H-16	139-L	8	2.9	5	90	9.7 ± 1.0	15.4 ± 1.2	18.1 ± 1.8	19.3 ± 0.1
3	Yreka	8	3.3	5	110	10.3 ± 3.1	14.5 ± 2.8	16.6 ± 3.3	19.3 ± 0.1
14	F 9650	2	3.3	1	90	8.1	11.3	15.4	17.7
18	0 9817-C	2	2.7	1	50	10.8	13.9	16.7	17.7
5	327	8	2.2	5	20	8.5 ± 1.0	13.2 ± 2.1	15.6 ± 2.3	17.3 ± 0.7
13	I-72	2	2.3	1	30	9.7	12.6	14.8	17.2
1	Shasta (H-1)	2	3.0	2	230	14.6	14.9	15.4	17.1
17	,, 412	2	2.6	1	240	6.5	11.2	14.6	16.7
2	,, (H-2)	2	2.2	2	140	13.5	13.6	14.3	16.5
10	Webster	2	6.5	2	1100	12.8	13.4	14.7	16.4
12	de Rosa	2	2.4	2	3300	12.8	13.4	14.2	16.2
19	—	2	3.5	1	70	7.0	9.7	12.1	15.5
15	F 9581	2	1.7	—	—	7.4	10.1	13.7	14.7
11	—	2	2.1	1	2200	6.7	9.5	11.3	13.6
								Mean	16.7 ± 0.4
C-11	Soemedang	2	8.4	2	†	3.3	6.4	7.9	8.2
5	TRU	2	20.0	2	†	3.3	7.0	7.7	8.0
7	53 H-1	2	9.3	2	†	2.9	6.0	6.6	7.0
4	K-120	2	23.0	2	†	2.0	4.4	5.1	5.2
K-1	A-1122	2	6.9	2	†	3.4	4.7	4.9	5.0
C-12	14	2	8.0	2	†	2.1	4.0	4.6	4.9
6	Tijwidej	2	8.8	2	†	1.6	3.8	4.4	4.7
3	Java	2	5.6	2	†	1.2	3.1	3.8	3.8
10	EV 76	2	7.6	2	†	1.3	2.8	3.4	3.6
8	A 1122	2	11.0	2	†	1.1	2.7	3.1	3.4
9	Bombay	2	8.5	2	†	0.6	1.8	2.2	2.4
								Mean	5.1 ± 0.5

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* LD$_{50}$—No. of organisms killing 50% of 8-14 weeks-old mice in 14 days. Reed and Muench.[8]

† 2×10^4 organisms injected subcutaneously into 8-12-weeks-old mice caused no deaths.

Catalase activity: ml of 0.1 N H_2O_2 decomposed in time interval shown.

[8] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

colorimeter using a green filter (540 mu) and a 15 mm cuvet. Five ml of this suspension were pipetted into the substrate solution (25 ml of 0.48 N H_2O_2 in 0.03 M Sorenson phosphate buffer at pH 6.80) in a 250 ml Erlenmeyer flask shaken at 100 oscillations per minute. At 5, 15, 30 and 60 minute intervals, 5 ml samples were removed to beakers containing 3 ml of 7 N H_2SO_4 and titrated with 0.1 N $KMnO_4$. Blank titration showed that no spontaneous decomposition of H_2O_2 occurred during the experiment. Enzyme activity is proportional to the quantity of H_2O_2 decomposed.

The viable bacterial count of the standard suspensions was determined by surface plating, and virulence was titrated by injecting 0.2 ml of appropriate dilutions subcutaneously into 8-14 week old mice, 10 mice being used for each of 3 serial dilutions.

Results. The results are presented in Table I. Inspection of Table I reveals that the catalase activity of virulent strains was significantly greater than that of avirulent strains. In the 14 virulent strains studied, the enzyme activity, as expressed in ml of 0.10 N H_2O_2 decomposed in 60 minutes, ranged from 12.9 to 19.9 ml with a mean value of 16.7 ml. On the other hand, for 11 avirulent strains, values of 2.1 to 8.6 ml with a mean value of 5.1 were found. The difference between the mean values of virulent and avirulent organisms is statistically significant.

Some of the factors involved in the catalase activity of *P. pestis* were investigated and the influence of a number of conditions was studied. It was observed in early experiments that growth on such different media as heart infusion broth (Difco), nutrient agar (Difco), or horse blood agar was without effect on catalase activity. The number of viable organisms was observed to undergo a 5-fold decrease when the incubation period was extended from 24 to 72 hours. Despite this decrease, no differences in catalase activity were exhibited by standard suspensions prepared from cultures after 24, 48, or 72 hours of incubation. The stability of the enzyme was also demonstrated by experiments in which standard suspensions were stored at 5°C for as long as seven days without change in activity. A final substrate concentration of 0.40 N H_2O_2 was found to be most suitable for our purposes. No significant differences in catalase activity were observed over a temperature range of 24-32°C or with changes in shaking rate from stationary to 103 oscillations per minute.

The kinetics of catalase activity in *P. pestis* under the conditions outlined appear to be similar to other findings[4-7] reported for cell-free preparations of this enzyme and apparently follow the curve for a monomolecular reaction (Table I).

The use of catalase activity as an *in vitro* screening test for virulence is suggested by the differences between virulent and avirulent strains. Experiments are being continued to determine whether this test will distinguish varying degrees of virulence during the *in vitro* attenuation of some of the virulent strains studied.

Summary. 1. Optimal conditions for catalase activity in *P. pestis* were determined and the catalase activity of 14 virulent and 11 avirulent strains was measured.

2. The catalase activity of virulent strains was significantly greater than that of the avirulent strains.

3. The possibility of using catalase activity as a screening test for determining virulence *in vitro* has been indicated.

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17097. Oxygen Consumption Studies with Intravenous Infusions of the Combined Fat Emulsion.*

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In previous reports from this laboratory, the results of experimental and clinical investigations of an intravenous fat emulsion combined with protein and glucose were presented.[1-3] The method of preparation of the combined fat emulsion and its effect upon humans were summarized in detail.[3] The present study is concerned with oxygen consumption associated with the intravenous infusion of the combined fat emulsion. Measured changes in oxygen consumption obtained with a variety of other intravenous preparations were made for purposes of comparison and served as control standards. This aspect of energy metabolism was the first of several methods of investigation with a view of obtaining evidence in favor of metabolic utilization of intravenously infused fat.

Method of study. Serial oxygen consumption determinations were conducted in a group of seven surgical patients who received one or more of 6 types of intravenous preparations. The patients, all males ranging in age from 18 to 58 years, were drawn from the surgical service. Because of good emotional stability, they were selected as the most suitable for oxygen uptake studies with the basal metabolism machine. Although these patients were on the surgical service because of a variety of diseases, at the time of testing there were no complicating factors of temperature elevation, active infection, chronic decubitus ulcers or acute organic metabolic disorders. Three different intravenous fat emulsions were studied; Emulsion 1, was a 5% fat emulsion which contained 50 g of coconut oil emulsified with 6% gelatin and provided 450 calories per liter; Emulsion 2, was a 10 per cent fat emulsion which contained 100 g of coconut oil emulsified with 6% gelatin to provide 900 calories per liter and Emulsion 3, always referred to in this paper as the combined fat emulsion, consisted of an homogenate of 10% coconut oil, 5% amigen, 5% glucose and 2% gelatin per liter with a caloric value of approximately 1300 calories. The comparative controls were (a) 5% glucose in normal saline, 200 calories per liter, (b) 6% gelatin solution, caloric value undetermined[4] and (c) 5% amigen and dextrose solution, 350-400 calories per liter.

Each infusion study was started in the morning with the patient in the post-absorptive state after a fasting period of 14 hours. In every case except one, a single liter of test fluid was administered. The duration of the infusion period ranged from 4 to 6 hours with 3 instances of extension up to 8 hours. Serial oxygen consumption determinations were obtained with the Benedict-Roth apparatus and on 2 occasions by the Tissot method. Each infusion study consisted of the determinations of the oxygen consumption level beginning with the pre-infusion period and measured in interval series during the infusion and post-infusion periods. The temperature, pulse, respirations and blood pressure were obtained throughout the study period at hourly intervals. Urine specimens were obtained at regular intervals for the first 14 hours and also at the end of 24 hours. Urinalyses were made for acetone (Rothera's test) and diacetic acid (Gerhardt's test).

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[3] Shafiroff, B. G. P., Mulholland, J. H., Roth, E., and Baron, H. C., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 343.

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TABLE I.
Oxygen Consumption Changes with Single Liter Intravenous Infusions of 5% Glucose in Normal Saline, of 6% Gelatin Solution, and of 5% Amigen-Dextrose Solution.

Type infusion	PT.		Oxygen consumption changes					
			Pre-inf.	Infusion period			Post-infusion period	
5% Glucose in N S	W B	at hr	0	2	4		5½	24
		cc/Sq M/min	127	121	117		119	126
		% change	0	—4.8	—7.9		—6.1	—0.4
	J S	at hr	0	2	4		5	
		cc/Sq M/min	123	128	124		116	
		% change	0	+4.0	+0.4		—5.7	
6% Gelatin	W B	at hr	0	2	4	5	7	
		cc/Sq M/min	126	120	123	127	124	
		% change	0	—5.2	—2.2	+0.4	—1.7	
5% Amigen in 5% Dextrose	J G	at hr	0	2	4	6		
		cc/Sq M/min	134	135	148	135		
		% change	0	+0.4	+10.0	+0.4		
	H K	at hr	0	2	6	8	10	12*
		cc/Sq M/min	140	136	156	139	144	163
		% change	0	—2.4	+11.2	—0.4	+3.4	+12.1

* ½ hour after a meal of 350 calories.

TABLE II.
Oxygen Consumption Changes with Single Liter Intravenous Infusions of 5% and 10% Fat Emulsions (50 and 100 g of Coconut Oil Emulsified with 6% Gelatin Solution).

Infusion fat emulsion	PT		Oxygen consumption changes							
			Pre-inf.	Infusion period			Post-infusion period			
5%	H K	at hr	0	2	4	6	8	10	12*	24
		cc/Sq M/min	152	183	186	179	160	140	178	154
		% change	0	+21.1	+23.4	+18.4	+5.8	—7.0	+18.3	+1.8
	J L	at hr	0	2	6	8	10	12	14	24
		cc/Sq M/min	113	137	145	154	145	132	122	116
		% change	0	+21.5	+27.6	+36.5	+27.6	+16.0	+7.7	+2.8
10%	J L	at hr	0	2	4	6	8	10	12	24
		cc/Sq M/min	129	147	152	173	147	130	129	126
		% change	0	+14.1	+18.0	+34.1	+5.8	+0.4	0	—1.9
	J S	at hr	0	2	4	6	8	10	12	24
		cc/Sq M/min	142	163	154	166	170	139	126	132
		% change	0	+14.4	+7.8	+16.8	+19.2	—2.3	—11.7	—7.0

* ½ hour after a meal of 400 calories.

Results. The oxygen consumption changes obtained with the 6 types of intravenous preparations are summarized in Tables I, II and III. The percentage changes in oxygen consumption during the infusion and post-infusion periods were computed for purposes of comparison with the initial or pre-infusion value taken as the zero level.

Maximal changes in oxygen consumption generally occurred after more than eighty per cent of any preparation had been infused or upon completion of the infusion period. With 5% glucose in normal saline and 6% gelatin infusions, the oxygen uptake values were mainly near the pre-infusion level while the recipients of 5% amigen and dextrose solution showed rises to about 11.0%. The recipients of 5% and 10% fat emulsions showed an increased oxygen consumption to levels ranging from 19.2 to 36.5% above their initial values. Elevations in oxygen consumption levels were usually greatest in recipients of the 10% combined fat emulsion with increases from 42.4 to 49.7%. One subject, FS, who received 2 liters of the 10% combined fat emulsion, was found to have in-

TABLE III.

Oxygen Consumption Change with Single Liter Intravenous Infusions of the 10% Combined Fat Emulsion (10% coconut oil, 5% amigen, 5% glucose and 2% gelatin solution).

		Oxygen consumption changes										
PT.		Pre-inf.	Infusion period				Post-infusion period					
W B	at hr	0	2	4			7					24
	cc/Sq M/min.*	167	221	243			193					152
	% change	0	+32.3	+45.4			+15.8					—9.1
H K	at hr	0	2	4			7					24
	cc/Sq M/min.*	167	209	225			171					152
	% change	0	+25.8	+34.8			+2.7					—9.1
J L	at hr	0	2	4	5		6	8	10	12	14	24
	cc/Sq M/min.	118	169	175	176		162	152	142	135	136	127
	% change	0	+43.8	+49.2	+49.7		+37.3	+28.9	+20.8	+14.9	+16.0	+7.4
S P	at hr	0	2	4	6		8					24
	cc/Sq M/min.	116	159	165	167		137					122
	% change	0	+36.8	+42.1	+43.3		+17.6					+6.4
S P	at hr	0	2	4	6		8	10	12	14		24
	cc/Sq M/min.	122	149	155	176		172	134	133	140		139
	% change	0	+20.2	+25.2	+42.4		+39.0	+8.8	+7.1	+12.6		+12.1
**	at hr	0	1½	3½	5	6¾		9½	12½			24
F S	cc/Sq M/min.	121	155	174	198	192		149	120			121
	% change	0	+28.3	+43.3	+63.9	+57.7		+23.2	—0.6			0
J S	at hr	0	2	4	6		8	10				24
	cc/Sq M/min.	124	148	165	179		178	168				137
	% change	0	+19.8	+33.8	+45.0		+44.1	+36.0				+11.3

* By Tissot method.

** FS received 2 liters.

creased his oxygen uptake to a maximum of 63.9% above his initial level. Another subject, HK was given oral feedings of approximately 350-400 calories at the 12th hour of each of 2 infusion studies as a result of which further elevations in oxygen uptake were again demonstrable.

Urinalysis for acetone and diacetic acid were positive in the intermediate serial samples in 3 patients who received the 5% or 10% emulsion. Tests for acetone were usually 2 and 3 plus in the 4 and 6 hour urine specimens followed by 1 plus to faint traces at 8 hours with negative results for the 10 hour and subsequent specimens. Tests for diacetic acid indicated faint traces only at the height of acetone spillage. Urine specimens were usually negative for ketone bodies in the 24 hour study period in the recipients of the 10% combined fat emulsion. In the latter group, the 4 hour urine specimen was found occassionally to contain a trace of acetone.

Temperature elevations ranging from 99.6 to 100.4° were noted between the tenth and sixteenth hours in the post-infusion period with the combined fat emulsion in the case of JL and SP. With the latter elevations in body temperature correlative secondary rises in oxygen consumption and moderate increases in pulse rate were found to occur. Analysis of temperature, pulse rate, respiration and blood pressure taken in each study at hourly intervals revealed minor fluctuations during the course of any type of infusion without significant findings associative with the oxygen consumption studies.

Comments. Evidence in favor of the metabolic utilization of intravenously infused fat has been reported by many investigators. The respiratory metabolism of human infants in relation to infusions of an homogenized fat emulsion was found to be reflected in increased heat production and alteration in the respiratory quotient. The energy exchange was derived from the combustion of the intravenously injected fat.[5] Other evidence in favor of utilization of intravenously infused fat has been obtained with parenterally administered C_{14} labelled tripalmitin emulsion as a result of which more than 50% of the radioactive isotope was recovered in expired carbon dioxide in a 24 hour study period. Proof was also given of incorporation of the labelled fatty acids into phospholipids.[6] In another experimental study data was presented to show that growing puppies obtained up to 30 per cent of their total energy requirement from daily infusions of a fat emulsion which in combination with a fat free diet provided for positive nitrogen balance and weight and growth gain.[7]

Indications of the metabolic utilization of a 10% combined fat emulsion administered intravenously were obtained by means of studies of oxygen consumption. For proper evaluation of the role of fat contained in the emulsion, 5 and 10% concentrations of the emulsified oil were studied. These studies showed that the increased oxygen consumption was not only correlative with fat as the prime contributing energy factor but that additive concentrations of fat raised significantly the amount of oxygen uptake. The non-fat components of the combined fat emulsion, gelatin, amigen and dextrose, were found to show small increments of change from the initial pre-infusion value. By combination of all the basic constituents with fat into a combined emulsion, the greatest increases in oxygen consumption resulted. Transient ketonuria noted in some of our studies were indicative both of early metabolic utilization and incomplete fat metabolism, the ketogenesis developing during the period of fat plethora.

The Benedict-Roth recording spirometer and the Tissot type apparatus were accepted by us as experimentally adequate for the present investigation. Alterations in tidal air as a possible explanation for increased oxygen consumption has also been eliminated in this study.[8]

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Summary. (1) Sixteen serial oxygen consumption studies were conducted in a group of 7 adult humans who received one or more of 6 types of intravenous preparations.

(2) Maximal increases in oxygen consumption above the initial pre-infusion levels attained their greatest values in recipients of the 10% combined fat emulsion when compared with a variety of intravenous preparations of a lesser caloric or energy value.

(3) With fat as the prime contributing factor to the increased energy value of the 10% combined fat emulsion, it is justifiable to conclude that the intravenously infused fat is utilized for energy production when in part it undergoes oxidative combustions which are ultimately measured in terms of oxygen consumed.

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17098. Effect of Dibenamine on Blood Flow and Cardiac Output in the Dog.

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Since N, N-dibenzyl-β-chloroethylamine (Dibenamine) was first introduced as an adrenergic blocking agent, many reports have been made on various aspects of its pharmacological properties.[1] Evidence has been presented indicating that Dibenamine produces vasodilation as evidenced by an increase in skin temperature,[2] and in dogs subjected to hemorrhage or trauma those pretreated with Dibenamine showed a slightly higher total blood flow than the controls.[3] It also has been reported that Dibenamine has no significant effect on cardiac output,[1] or increases cardiac output,[4] although no data are given on this point. In this report data are presented on the effects of Dibenamine on blood flow and cardiac output.

Methods. With but one exception the dogs were anesthetized with barbital sodium 300 mg/kg, chloralose, 250 mg/kg, was used in one dog. Blood pressure was obtained from a carotid artery with a Hamilton manometer and cardiac output determined from the pressure pulse by the method of Hamilton and Remington.[5] Blood flow was recorded from either a carotid or a femoral artery by means of a rotometer.[6] The anticoagulant used was chlorazol fast pink, 2 cc of an 8% solution per kg. The dose of Dibenamine, 20 mg/kg, when given intravenously (i.v.), was administered slowly over a 5-minute period. In a majority of the dogs the degree of adrenolytic action was tested with epinephrine 30 minutes after the Dibenamine injection. In all cases only a depressor response was elicited.

Results. Table I summarizes the data on those dogs in which cardiac output, blood pressure and blood flow were determined; pressure and flow were measured in 16 additional dogs. In all of the 24 dogs but 7 the blood pressure dropped significantly, 3 of these showed a rise in pressure and in the other 4 the changes were less than 10 mm Hg. The drop in pressure occurred about 20 minutes after Dibenamine was given. Little effect was noted on heart rate with a slight

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TABLE I.
Effects of Dibenamine on Blood Pressure, Cardiac Output and Peripheral Blood Flow.

	Blood pressure			Cardiac output, liters/M^2			Blood flow, cc/min.		
		After Dibenamine			After Dibenamine			After Dibenamine	
Dog	Control	30 min.	60 min.	Control	30 min.	60 min.	Control	30 min.	60 min.
DF 13	207/175	155/105	135/60	2.5	3.0	4.8	82	45	41
3	150/128	93/75	91/25	1.5	1.45	1.7	4	8	4
14	190/135	198/112	202/140	3.4	4.0	3.8	60	116	135
15	295/242	102/57	Died	6.0	4.7	Died	148	16	Died
16	190/157	95/77	95/70	1.4	1.3	1.3	82	32	12
17	185/162	130/115	122/107	1.2	1.0	1.0	39	36	41
18	205/180	155/130	152/110	1.9	0.9	2	55	28	30
19	220/187	150/87	145/77	1.8	3.9	3.4	48	39	36

increase being the most frequent response. The blood flow during the experimental period, dropped in 16, remained the same in 6 and increased in 2 dogs one of which had a blood pressure rise of 12 mm Hg and the other a fall of 10 mm Hg. The flow in the other 2 dogs, whose pressure was increased, remained constant in one and dropped 30% in the other. The cardiac output dropped in one case only (DF 15), there was an increase in 2 (DF 13, DF 18), and the others did not change significantly.

Dibenamine was administered intra-arterially in 2 to 4 mg/kg amounts in 3 dogs. There was no evidence of any peripheral action.

In 2 dogs epinephrine, 0.01 mg/kg was given at the end of the 30 minutes period, the cardiac output approximately doubled returning to the control level in from 2 to 3 minutes, the heart rate increased between 10 and 15% and the systolic and diastolic pressure fell.

No detectable difference in response was noted in the dog receiving chloralose.

Discussion. Dibenamine in adrenolytic doses produces a significant drop in blood flow to the head or leg, but has little effect on cardiac output of the dog. The drop in blood pressure and the change in the pressure pulse are probably the result of a vasodilation in the splanchnic area which does not include the kidney.[7]

Summary. Following Dibenamine, cardiac output remained essentially unaltered in 5 of 8 dogs, peripheral blood flow decreased significantly in 16 of 24 dogs.

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[7] Wang, C., and Nickerson, M., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 92.

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17099. Effect of an Antagonist of Pteroylglutamic Acid on the Leukemia of Ak Mice.*

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In a previous paper[1] hematologic and histologic findings in mice with an induced deficiency of pteroylglutamic acid (PGA) were described, together with a method for producing the deficiency with the aid of a crude chemical antagonist of PGA (so-called "x-methyl PGA," presumably a mixture of 7-methyl- and 9-methyl PGA).†

Following this study we became interested

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[1] Weir, D. R., Heinle, R. W., and Welch, A. D., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 211.

in the influence of this deficiency on the transplanted leukemia of Ak mice. About 75% of this strain develop spontaneous leukemia after the age of 6 months. The intravenous injection of leukemic cells from diseased older animals produces progressive leukemia in 100% of younger animals.[2] It was originally planned to make the transplant after the deficiency, as evidenced by leucopenia, had been established. This approach was found to be unsuitable, probably because the Ak strain is not hardy enough to survive the dietary regime for the preliminary 6 to 8 weeks required for the development of the deficiency, together with the additional time necessary for the growth of the transplanted material. Accordingly, it was decided to transplant the leukemic cells about 15 days after the start of the experiment.

In a preliminary experiment there had been some indication that the parenteral administration of the crude synthetic antagonist of PGA (Lederle N-67A)[†] might be more effective than its oral administration. Accordingly, for comparative study a microbiologically assayed solution of partially purified "x-methyl PGA" was used.[‡] The preliminary work also indicated the desirability of pair-feeding the animals in an attempt to rule out the possible effects of inanition as a result of diminished intake of PGA.

Methods. Weanling Ak mice weighing with-

TABLE I. Results with Purified Diet and Added Oral and Parenteral Supplements.

Group	Leukemic animals (see text)	Supplements to purified diet	Survival after transplanting			Leucocytes		"Blasts"	Histological findings		
			Mean days	Max. days	Min. days	Max. daily mean, per cmm	Max. single count, per cmm	Max. daily mean, per cmm	Liver	Spleen	Marrow
1	7	Parent. antagonist	46	71	27	38,000	55,000	1026	++	+++	+++
2	4	Oral antagonist Parent. antagonist Parent. PGA	38	73	22	34,500	55,000	1380	+++	++++	++++
3	4	Oral antagonist Parent. antagonist	53	77	35	14,750	27,000	140	0	+	++
4	4	Oral antagonist Parent. Locke's sol.	45	63	19	17,000	23,000	97	0	0	++
Controls	5	Normal diet No supplement	19	23	16	92,000	108,000	4600	++++	++++	++++

[†] The crude antagonist was prepared by Dr. M. E. Hultquist and Dr. J. M. Smith, Jr., Calco Chemical Company, by reacting 2,4,5-triamino-6-hydroxy-pyrimidine and p-amino-benzoyl-1(+)-glutamic acid with 2,3-dibromobutyraldehyde in the reaction described by Angier *et al.*, *Science*, 1946, **103**, 667. We are much indebted to Dr. T. H. Jukes of the Lederle Laboratories Division, American Cyanamid Company, who supplied us with this antagonist of PGA.

[2] Weir, D. R., and Heinle, R. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 268.

[‡] Assayed on *Streptococcus faecalis R*, each ml of the solution used contained the equivalent of 68 mg of a crystalline reference standard supplied by Dr. E. L. R. Stokstad of the Lederle Laboratories. However, it developed subsequently that this microbiologically active fraction is not responsible for the effect of the crude antagonist on animal species.

in 5 g of each other were arranged in 4 groups of 10 animals each and one group of 5 animals (Table I). The composition of the purified diet has been given previously.[1] In this experiment the diet included 1% succinylsulfathiazole from the beginning. All groups were maintained on this diet except the controls. For Groups 2, 3, and 4, 0.5% powdered antagonist was added to the diet. On the 13th day parenteral therapy was started. One cc of the antagonist solution was given daily intraperitoneally to Groups 1, 2, and 3; 20 μg of PGA in 0.2 ml of Locke's solution was given daily subcutaneously to Group 2; and 1 cc of Locke's solution was given daily intraperitoneally to Group 4. On the 16th day all animals were transplanted. The possibility that the potency of the cell suspension might deteriorate during the time of inoculation of a large group of animals was taken into account. Animals from the various groups were taken in rotation so that no one entire group was inoculated either at the beginning or end of the transplanting period.

The animals of groups 2 and 4 were fed the same amount of diet as that consumed on the previous day by the animals of Group 3 (thus, animals No. 11 and No. 31 were fed the amount of food consumed by animal No. 21 on the previous day). Twice weekly all animals were weighed and total and differential leucocyte counts were done. At death, sections of liver, spleen, and bone marrow were prepared for histologic studies.

Results. As in the preliminary experiments it was found that the Ak animals do not survive well under these forms of treatment; many mice died before the leukemia could have developed. Others died during the period when the controls showed florid leukemia, but did not themselves show any evidence of the disease, either by blood counts or by gross or microscopic pathologic examination. Therefore, it was considered justifiable to eliminate from consideration those animals which died early or which did not show any evidence of leukemia at any time. In Group 1, 7 animals remained and in each of Groups 2, 3, and 4, 4 animals remained. All 5 of the controls were dead of leukemia within 23 days after transplanting. Although a reliable demonstration of the effect of the regime on time of survival was prevented by the early mortality of many of the experimental animals, other findings are of considerable interest. The results of the experiment are shown in Table I.

The control animals developed the typical picture of the transplanted disease; all had very high leucocyte counts with blast cells in the peripheral blood, and histologic sections showed extensive leukemic infiltration in the liver, spleen, and marrow. Although all controls were dead within 23 days, the mean survival time of the experimental groups was about 46 days. Control animals with transplanted Ak leukemia almost invariably die within a few days of each other, a circumstance which obtained in this experiment. In our experience the interval between inoculation and death may vary in different experiments, due probably to variation in the donors, but in any one experiment the time of survival of various individuals differs only by a few days. Accordingly, the apparent increase in the mean survival time in the experimental groups was considered to be encouraging in import.

The animals in Group 4 which received the diet and oral antagonist showed the maximal alteration of the leukemic process. The white blood cell counts never rose above the maximal normal of 24,000 per cmm and only small numbers of blast cells appeared in the peripheral blood. Histologically the livers and spleens of these mice showed no leukemic infiltration and the marrows showed only moderate infiltration. Essentially the same picture was seen in Group 3, indicating that the action of the oral antagonist was not significantly altered by the parenteral preparation. This conclusion was supported by the finding that the animals in Group 1, which received the parenteral antagonist without oral antagonist, showed a moderate increase in white blood counts, moderate numbers of blasts, and histologic findings which differed very little from the controls. The animals in Group 2 received the diet with both oral and parenteral antagonist, supplemented with parenterally administered PGA. It had been calculated that a daily dose of 20 μg of PGA would combat all effects of the antagonist. Unfortunately this was not true. However, the

TABLE II.
Results with Normal Mouse Diet and Antagonist Added in Excess.

			Survival after transplanting			Leucocytes		
Group	No. of animals	Diet supplement	Mean days	Max. days	Min. days	Max. daily mean, per cmm ×1000	Max. single count, per cmm ×1000	"Blasts" max. daily mean, per cmm
1	8	No antagonist	16.1	21	14	82	127	1,800
2	8	2% PGA-antagonist	39.0	72	31	45	88	4,200
3	8	4% " "	57.0	74	41	22	32	1,600

dose was sufficient to allow the development of moderately high white counts with moderate numbers of blasts and histological changes which could not be distinguished from the controls.

Within each group the histologic changes were surprisingly uniform in the individual animals. The plus values given in Table I apply to each individual animal and are not means for the group.

In all the experimental groups growth was arrested and there was a steady loss of weight. In this respect none of the groups differed from the others.

Because of the high mortality of the experimental animals on the purified diet, as described in Part I of this experiment, the effect of the PGA-antagonist, when added to an unpurified diet known to be nutritionally adequate for the mouse (Purina dog chow), was studied.

Methods. In preliminary experiments, PGA-antagonist at levels of 0.5% and 1.0% had had some effect on the progress of the leukemia but not enough to be statistically significant. Accordingly, 24 Ak mice, weighing within 5 g of each other, were arranged in 3 groups of 8 animals each and were fed Purina dog chow ground into a coarse powder. Group 1 (controls) was given no supplement, while Group 2 and Group 3 were given the antagonist at levels of 2 and 4%, respectively. The animals of Groups 1 and 2 were fed that amount of diet consumed by Group 3 on the previous day. Twice weekly all animals were weighed and total and differential leucocyte counts were done. On the fifteenth day all animals were transplanted. At autopsy, sections of liver, spleen, and bone marrow were made for histologic study.

Results. In contrast to the experience described in Part I, the dietary regimen of Part II was very well tolerated by all animals. None of the animals died in the preliminary stages and all developed evidence of leukemia before death. The results are given in Table II. With 2% antagonist the mean survival time was more than doubled, and with 4% it was more than tripled. In neither of the supplemented groups did an animal expire until at least 10 days after the last control animal had died.

The peripheral blood of the experimental animals showed more evidence of leukemia than was seen in the experimental animals on the purified diet of Part I. Even with PGA-antagonist at a level of 4% many blast forms appeared although only 2 total leucocyte counts were above 24,000, the maximal normal.

In contrast to the animals on the deficiency regimen of Part I, the histologic findings in the experimental animals of Part II were indistinguishable from those of the controls. Infiltration of all organs was extensive. Very probably this infiltration occurred later and more slowly in the experimental animals as compared to the controls.

There was a moderate loss of weight in some animals; others gained. The mean loss was considerably less than that shown by the animals on the deficiency regimen.

Discussion. Several factors might be considered as having contributed to the experimental results; among these are: 1) simple inanition, 2) deficiency of PGA, 3) an effect of one or more substances in the crude antagonist. Inanition might be seriously considered, if the increased survival times alone were taken into account, and if the paired feeding technic did not rule out inanition as the determining factor.

If a profound, generalized deficiency of PGA were the determining factor one might expect some evidence for this in the leucocyte picture of the peripheral blood of the experimental animals at the time the controls had florid leukemia. However, no such evidence was found. In this experiment the leucocyte counts were not low at that time and in a preliminary experiment, run under similar conditions, there were no changes in either the white or red blood cell counts. Also, it has been shown in previous experiments[1] that hematologic and histologic evidence of PGA-deficiency do not develop until the animals have been on a purified diet containing succinylsulfathiazole and antagonist for 50 to 60 days. The rate at which PGA-deficiency might develop without the purified diet, as in Part II, has not yet been determined. Of course these findings do not mean that a selective or relative deficiency may not exist. It seems likely that leukemic cells may require a much more abundant supply of PGA than do normal cells.[3] A moderate decrease in available PGA, not enough to produce leucopenia in a normal animal, might be sufficient to decrease the rate of development of leukemic cells. On the other hand, except for the blasts, the leucocytes in the peripheral blood have the appearance of normal cells and merely exist in greater numbers. Perhaps the relative deficiency acts by limiting the excessive production of these essentially normal cells, rather than by interfering with the metabolism of abnormal cells. This reasoning may apply to the cells in the peripheral blood, but does not necessarily explain the marked reduction or complete absence of leukemic infiltration in the liver, spleen, and marrow of the experimental animals in Part I. The infiltrating cells are defintely abnormal as are the blasts of the peripheral blood.

If PGA-deficiency were not the sole explanation for these findings, it is conceivable that an active substance other than the antagonists of PGA may be present in the crude antagonist. However, all growth experiments have indicated that the effects of this material are reversed completely by synthetic PGA.[4,1] Further experiments designed to determine whether PGA-deficiency can account completely for the observed inhibition of the leukemic process are now in progress. These experiments have shown that the leukemic process in mice can be controlled partially by methods and substances now available. Life is prolonged and the severity of the disease is reduced. It is apparent that with the technics so far devised the effects are not of permanent value and the effect on the normal metabolic activity of the animal can be severe. If a profound deficiency of PGA is produced, the leukemia may be controlled, but the treatment eventually is as fatal as the disease. It is logical to expect that this might be true, for it may be assumed that PGA is an essential metabolite utilized in the manufacture of many types of cells and the requirement for the formation of abnormal leucocytes, though larger, probably is not inordinately greater than that for normal cell formation. That the animals of Part I, given a purified diet, developed a deficiency of PGA in the terminal state, is certain. However, additional studies will be required to prove that here also a deficiency of PGA was the only factor operating in the early stage of the experiment when leukemia was suppressed in the experimental groups while the controls had developed florid leukemia. It is our hope that a regimen may be worked out which will provide for selective inhibition of the leukemic process without too seriously impairing the processes essential to the life of normal cells.

Summary. The transplanted leukemia of Ak mice can be partially controlled either by a pteroylglutamic acid-free diet, combined with succinylsulfathiazole and a crude antagonist of pteroylglutamic acid, or by the addition of large amounts of the crude antagonist to an unpurified "normal mouse diet." Life is prolonged and the severity of the disease is reduced.

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[3] Bethell, F. H., and Swendseid, M. E., *J. Clin. Invest.*, 1946, **25**, 917.

[4] Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H., *J. Biol. Chem.*, 1947, **169**, 427.

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17100. Lymphopenia and the Secretion of Adrenalin.*

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It was shown in a preceding paper[1] that the injection of adrenalin in doses of from 0.05 to 1 $\gamma/100$ g significantly lowers the lymphocyte count of the rat while the number of neutrophils is reduced to a lesser extent. This result (lymphopenia and a relative neutrophilia[†]) is not seen in adrenalectomized animals. The great sensitivity of this reaction makes it probable that it may occur under conditions involving not the injection but the secretion of adrenalin. Since the question of increased adrenalin secretion in physiological conditions is still controversial (See Rogoff,[2] for the older literature see Gellhorn[3]) it was decided to investigate the action of hemorrhage, emotional excitement and insulin hypoglycemia on the lymphocyte count. Elmadjian and Pincus[4] showed already that struggle (excitement) and exposure to cold resulted in lymphopenia and that this effect was absent in adrenalectomized rats. However the experiments of these authors did not deal with the question whether the secretion of adrenalin formed a necessary link in the chain of events leading to lymphopenia under conditions of struggle, etc. The present work was undertaken to fill this gap.

Method. The experiments were performed on adult Sprague-Dawley rats (weight 150 to 200 g) after a 16 hour fast unless otherwise stated. Blood samples were taken from the tail blood and the lymphocytes were determined before and 20, 40 and 60 minutes after the experimental procedure. Three procedures were used. The first consisted in hemorrhage (1 cc blood/100 g weight removed from the tail veins); the second involved emotional excitement induced by a slight electric shock which elicited a "rage" reaction but did not produce a convulsion. As a third procedure insulin[‡] was injected intraperitoneally and the effect of varying degrees of hypoglycemia was studied. The blood sugar was determined as in previous papers.[5] The experiments were performed on normal and adrenodemedullated rats. The latter were used not earlier than 10 to 14 days after operation and only after a test with adrenalin 0.1 $\gamma/100$ g i.p. had resulted in a marked lymphopenia. If this effect was not clear cut the animal was either discarded or used after a second adrenalin test taken one or two weeks later had proved the responsiveness of the adrenal cortex to injected adrenalin.

Results. I. *The effect of hemorrhage on the lymphocyte count.* Table I shows the percentage changes in lymphocytes and polynuclear leucocytes following hemorrhage. In 60 minutes the former fell to 66.9% of the control value in normal rats whereas adrenodemedullated animals showed only an insignificant fall (to 91.5%). This difference is statistically significant ($P < 0.01$). The polynuclear cells fell only slightly. As the red cell count indicates the difference existing between the normal and adrenodemedullated rats is not accounted for by differences in the dilution of the blood and must therefore be attributed to a reaction dependent on the secretion of adrenalin. The fact that the reaction observed in normal rats has the same characteristics (lymphopenia and relative neutrophilia) as seen after injection of adrenalin supports this conclusion.

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[*] Aided by a grant from the Office of Naval Research.

[1] Gellhorn, E., and Frank, S., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 426.

[†] Through an oversight this effect was described in the preceding paper as lymphopenia and neutrophilia instead of lymphopenia and *relative* neutrophilia.

[2] Rogoff, J. M., *J. Genetic Psychol.*, 1945, **32**, 249.

[3] Gellhorn, E., *Autonomic Regulations*, New York, 1943.

[4] Elmadjian, F., and Pincus, G., *Endocrinology*, 1945, **37**, 47.

[‡] Kindly supplied by the Eli Lilly and Co.

[5] Safford, H., and Gellhorn, E., Proc. Soc. Exp. Biol. and Med., 1945, **60**, 98.

TABLE I.

A. Effect of Hemorrhage on the Blood Count of the Rat.*

	Lymphocytes in %				Neutrophils in %				Neutrophils / Total leucocytes				P†	% of red blood cells at 60′
	0′	20′	40′	60′	0′	20′	40′	60′	0′	20′	40′	60′		
Normal	100	85	78	67	100	99	84	80	100	110	114	121		94
Adrenodemedullated	100	91	95	92	100	83	92	86	100	95	100	96	<.01	96
	B. Effect of the Rage Reaction on the Blood Count.													
Normal	100	76	55	46	100	88	84	72	100	114	127	131		101
Adrenodemedullated	100	91	84	82	100	101	100	103	100	110	116	116	<.001	98

* Each group consisted of 10 rats which were fasted 16 hours prior to the experiment.
† Probability coefficient applied to the lymphocytes in the 2 groups at 60 minutes.

II. *The effect of "rage" on the lymphocyte count.* Normal and adrenodemedullated rats were given a slight electroshock applied to both ears for 0.4 second. The rats reacted strongly to the stimulus with struggling and vocalization but were quiet a few minutes later. The effect on the lymphocytes was similar to that described in the first group but the difference in the reaction between normal and adrenodemedullated rats was even greater ($P < 0.001$. The change in the neutrophil count was again much less than in that of the lymphocytes, leading to a lymphopenia and a relative neutrophilia as on injection of adrenalin. No changes occurred in the neutrophils of the demedullated group and the fall in the lymphocytes was very slight. The red cell count before and 60 minutes after the "rage" reaction showed clearly that the changes in the lymphocytes of normal rats in conditions of "rage" are not due to a dilution of the blood.

III. *The action of hypoglycemia on the lymphocyte count.* A series of experiments listed in Table II was performed to determine the action of insulin hypoglycemia on the lymphocyte count of normal and adrenodemedullated rats. Since the latter are more sensitive to insulin than normal rats the experimental conditions had to be slightly modified for the two groups in order to obtain similar degrees of hypoglycemia. Table II shows that this was accomplished by varying the time interval of insulin injections and the duration of fasting. If both groups (normal and demedullated rats) were given 2 successive injections of 0.5 unit/kilo insulin i.p. at an interval of 20 minutes the demedullated rats were in coma at about 30 to 40 minutes but the normal rats at 60 minutes. By delaying the second injection by 10 minutes the demedullated group went into coma at about the same time as the unoperated group. As the Table shows the effect of insulin on blood sugar was very similar in groups A (normal) and D (demedullated); likewise the mild hypoglycemic effects in groups C and E were about the same. In the groups A and D in which each animal was in coma at 60 minutes the average lymphocyte count was 60 and 70.2% respectively of the preexperimental level in the normal and the

TABLE II.

A. Effect of Insulin Hypoglycemia on the Blood Count of the Rat. Dose 0.5 μ/kg.

Group	Hrs fasted	Dose	Blood sugar at 60′	% of lymphocytes				% of neutrophils				Neutrophils / Total leucocytes			
				0′	20′	40′	60′	0′	20′	40′	60′	0′	20′	40′	60′
A. Normal*	36	at 0′ and 20′	28.3 ± 8.48	100	83	73	60	100	91	83	76	100	112	122	136
B. ,, †	16	at 0′ and 20′	43.3 ± 6.39	100	83	77	70	100	89	84	83	100	107	115	123
C. ,,	16	at 0′	50.2 ± 4.96	100	89	83	75	100	93	83	80	100	106	106	112
D. Adrenodemedullated	24	at 0′ and 30′	31.8 ± 9.6	100	82	75	72	100	94	84	87	100	101	102	107
E. ,,	16	at 0′ and 20′	46.4 ± 14.97	100	92	85	79	100	92	85	88	100	98	99	113

* Group A and D consisted of 11 animals.

† Group B, C, and E consisted of 10 animals.

B. Significance of the Changes in Lymphocytes.

Groups compared	P*
A, C	<.01
A, D	<.05
D, E	<.2
A, B	<.05
C, D	<.9
C, E	.5

* Probability coefficient applied to lymphocytes in the two groups at 60′.

C. Effect of Insulin Hypoglycemia on the Blood Count of the Adrenalectomized Rat.*

Blood sugar at 60′ (mg%)	% of lymphocytes				% of neutrophils				Neutrophils / Total leukocytes				% red blood cells at 60′
	0′	20′	40′	60′	0′	20′	40′	60′	0′	20′	40′	60′	
31 ± 2.9	100	101	98	90	100	101	95	107	100	99	98	111	101%

* Insulin 0.2 μ/kg was injected in 10 rats fasted for 16 hours and resulted in deep coma.

demedullated group. The statistical evaluation shows that P is < 0.05 suggesting probable significance. As the hypoglycemic effect diminishes from A to C in the normal group (the final, 60 minutes—blood sugar being 28, 43 and 50 mg % respectively) the lymphopenia becomes progressively less (60% in group A, 70% in B and 75% in C). Comparison of A to C on the basis of the lymphocyte count at 60 minutes gives $P < 0.01$ indicating that the difference is statistically significant. On the other hand similar differences in hypoglycemia in the demedullated group (D and E) do not lead to statistically significant differences in lymphocyte count. These data suggest that in hypoglycemia the increased secretion of adrenalin contributes to lymphopenia. However further control experiments appeared in order. Therefore a final group of insulin tests was performed on adrenalectomized rats in which the count of red cells and lymphocytes, and the blood sugar were determined. As Table II shows adrenalectomized rats show only a very slight fall in lymphocytes and no characteristic changes in neutrophils. In view of the fact that the lymphocytes fell more in demedullated than in adrenalectomized animals subjected to insulin coma it is suggested that in addition to the lymphopenia initiated by the increased secretion of adrenalin some other effect of low blood sugar on the adrenal cortex occurs which leads to lymphopenia without the intervention of functional changes in the secretion of adrenalin. Here again the red blood count remained unchanged.

Our investigations show that hemorrhage, general ("emotional") excitement and, to a lesser extent, insulin hypoglycemia lead to a lymphopenia and a relative neutrophilia in normal but not in adrenodemedullated rats. As was shown previously similar changes may be induced by the injection of adrenalin. Since hemorrhage, general excitation and injection of adrenalin no longer cause these effects in adrenalectomized rats it must be concluded that emotional excitement and hemorrhage lead to an increased secretion of adrenalin which indirectly, probably through a decrease in the level of cortical hormones in blood and tissues (Sayers and Sayers,[6] Gellhorn and Frank), leads to a discharge of adrenocortical hormones resulting in lymphopenia. Insulin hypoglycemia causes a similar reaction but our experiment suggests an additional action of hypoglycemia on the hypophyseal-adrenocortical system since hypoglycemia acts much more on the lymphocyte count of adrenodemedullated than on that of adrenalectomized rats. This action appears to be the cause of the lesser difference between adrenodemedullated and normal rats under conditions of hypoglycemic coma than was found between these groups of animals subjected to hemorrhage and excitement. Observations by Parsons and collaborators[7] on the action of insulin hypoglycemia and injection of adrenalin on the blood count of schizophrenics agree with our present and previous experiments except for the fact that in these experiments lymphopenia is associated with an absolute neutrophilia whereas in our work on rats injection or secretion of adrenlin leads to lymphopenia with relative neutrophilia. However, this difference is probably accounted for by the fact that adrenalin in large doses causes lymphopenia and neutrophilia (Hungerford).[8]

Summary. Hemorrhage (1 cc/100 g body weight) and general excitement (induced by the action of a subconvulsive electrical shock) cause lymphopenia and relative neutrophilia in normal but not in adrenodemedullated rats. Insulin hypoglycemia leading to coma induces a greater lymphopenia in normal than in adrenodemedullated animals. It is concluded that hemorrhage, general excitement and insulin coma cause an increased secretion of adrenalin followed by an increased discharge of adrenocortical hormones. Hypoglycemia seems to induce some increased adrenocortical secretion even in the absence of the adrenal medulla.

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[8] Hungerford, G. F., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 356.

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17101 P. Interaction of Swine Influenza Virus and Egg-White Inhibitor of Virus Hemagglutination.*

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The interaction of swine influenza virus and egg-white (EW) inhibitor of virus hemagglutination resembles an enzymatic reaction with the virus acting as enzyme and the inhibitor as substrate.[1] Attempts to follow the kinetics of inhibitor inactivation by virus have revealed that the inhibitor present during the intermediate period of the reaction differs in behavior from untreated inhibitor. This observation and its possible significance are the subjects of the present report.

Materials and Methods. The swine influenza virus was the purified preparation SF, previously described,[1] which contained about 7,200 hemagglutinating doses (HD) and 0.234 mg N per ml. The preparation of semipurified inhibitor, A200 PII EI, was obtained by precipitating EW with 7 volumes 0.1 M KH_2PO_4 and extracting the precipitate, after washing, with 0.06 M phosphate buffer at pH 7.2.[2] The extract contained 120 γ N per ml and was about 30 times as active as EW on a N basis. The residual inhibitory activity of virus-inhibitor mixtures was titrated against 4 HD heated swine virus (53°C, 30 minutes) by the constant virus-varying inhibitor method.[2] Preliminary to titration, the virus-inhibitor mixtures were immersed in boiling water for 2 minutes to destroy the hemagglutinating activity of the virus; the activity of the inhibitor was not significantly affected by this treatment. Buffered saline, consisting of 0.81% NaCl and 0.005 M phosphate at pH 7.3, was the diluent throughout.

Experimental. In a typical experiment, mixtures of equal volumes of 1:5 inhibitor and 1:200 SF were incubated for varying periods at room temperature (27°C), immersed in boiling water, cooled, and titrated for residual inhibitory activity with virus heated at 53°C; inhibitor controls devoid of virus were included. The complete titration data are shown in Fig. 1, in which both the conventional designations, O to ++++, denoting minimal to maximal hemagglutination, and the corresponding chicken red blood cell concentrations in the reference red-cell suspensions are used as ordinates. In the present experiment, the designation +++± corresponds to zero inhibition. From the results it is evident that virus-treated inhibitor cannot properly be described as diluted untreated inhibitor, since the inhibition curves cannot be superimposed by translation along the axis of inhibitor dilution; therefore, no simple kinetic analysis is possible. Hirst[3] has re-

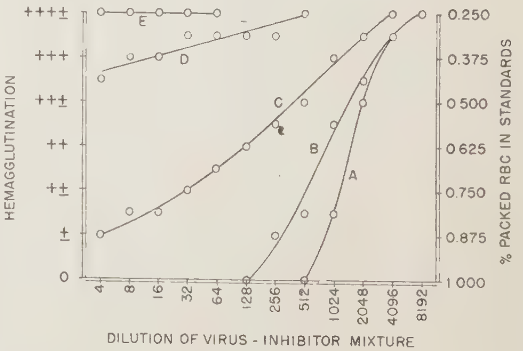

Fig. 1.

Inhibitory activity of purified EW inhibitor (A200 PII EI) after incubation with purified swine influenza virus (SF) for varying periods at 27°C, as tested with heated (53°C, 30 minutes) virus. Period of incubation: curve A, 0 minutes; B, 3 minutes; C, 10 minutes; D, 30 minutes; E, 100 minutes. Curve A applies also to untreated inhibitor control and to inhibitor control heated in boiling water for 2 minutes.

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* This investigation was supported by a grant to Duke University from Lederle Laboratories, Inc., Pearl River, N. Y., by the Dorothy Beard Research Fund and by a research grant from the National Cancer Institute, U. S. Public Health Service.

[1] Lanni, F., and Beard, J. W., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 442.

[2] Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W., *J. Biol. Chem.*, in press.

[3] Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 315.

ported a similar result with normal serum inhibitor.

Experiments to clarify the origin of the progressive decrease in slope induced by virus (Fig. 1) have shown that (a) the slope effect is not attributable to the concentration of impurities (cf. 2) or to the accumulation of noninhibitory reaction products; and (b) the inhibitor characterized by shallow slope does not sediment appreciably in centrifugal fields sufficient for sedimenting virus.

Discussion. The slope of the inhibition curves (Fig. 1) in the region of transition from zero to maximal agglutination may be tentatively interpreted in terms of the firmness of attachment of inhibitor to the particles of the *titrating* virus. Accordingly, virus-treated inhibitor may be described as a "weak" inhibitor in comparison with untreated inhibitor.

Available evidence suggests that the weak inhibitor is a free inhibitor, unattached to virus. Such an inhibitor could pre-exist in a heterogeneous inhibitor population and could be made manifest through a preferential action of virus against strong inhibitor. An attractive alternative is that the weak inhibitor is the product of a progressive, rather than all-or-none, action of virus. If the latter explanation should be correct, the demonstration of a free, weak inhibitor, intermediate between fully active and inactive inhibitor, would provide strong support for the enzymatic hypothesis of inhibitor inactivation by virus. The possibility that the action of virus is not all-or-none has been previously suggested by Burnet and collaborators[4,5] from related, but somewhat more complicated, experiments.

Summary. During the interaction of swine influenza virus and egg-white inhibitor of hemagglutination, there appears a weak inhibitor, which has titrating properties different from those of untreated inhibitor. This result renders impossible a simple kinetic treatment of the data. The significance of the weak inhibitor for the mechanism of virus-inhibitor interaction is discussed.

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17102. Role of Adrenal in Uptake of I^{131} by the Thyroid Following Parenteral Administration of Epinephrine.

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In a previous paper[1] we reported that the parenteral injection of epinephrine into both the intact and the totally thyroidectomized dog resulted in an increase in secretion of thyrotropin from the adenohypophysis. In the intact dog this was evidenced by the development of hyperplastic changes in the thyroid following the daily injection of adrenalin-in-oil. Serum obtained from similarly treated totally thyroidectomized animals, when injected subcutaneously into young guinea pigs not exceeding 200 g in weight, resulted in hyperplastic changes in the thyroids of the treated guinea pigs.

It was further reported that the increase in circulating thyrotropic factor resulting from the injection of epinephrine in totally thyroidectomized dogs reached its peak approximately 4 to 6 days following the beginning of treatment, and thereafter began to diminish despite the continued injection of epinephrine.

The present report is concerned with a study of the role of the adrenals in the above described phenomena. In place of the biological assay method previously employed for the determination of circulating thyrotropin, we used the percentage uptake of parentally administered I^{131} as an index of thyroid activity. This technique has the relative advantages of simplicity and a higher degree of accuracy over the biological assay method.

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[1] Soffer, L. J., Volterra, M., Gabrilove, J. L., Pollack, A., and Jacobs, M., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 446.

Methods. Male rats of the Wistar strain (Carworth Farms) weighing approximately 125 g were employed. They were placed on a low iodine, minimally goitrogenic (Steenboch) diet[*] for at least 3 weeks before being used experimentally in order to insure an adequate uptake by the thyroid gland of I^{131}.[2] In the adrenalectomy experiments, rats of the Sprague-Dawley strain were also employed because of the reported lesser frequency of accessory adrenal tissue.

The experiments were planned to study the 24-hour percentage uptake of parenterally administered I^{131} by the thyroid gland after the administration of epinephrine in the intact and in the adrenalectomized animals. Some preliminary studies were conducted to determine the influence of the daily injection of 17-hydroxy-11 dehydrocorticosterone[†] upon the response of the bilaterally adrenalectomized animals.

All experiments were conducted with litter mates acting as controls. The animals in each experimental group were subjected to the same diet and environment and for the same duration of time, although the period of time that the respective groups were kept on the low iodine diet varied from experiment to experiment. In no instance, however, was such a period less than 3 weeks.

In the intact animals epinephrine was administered twice a day at 9:00 A.M. and 5:00 P.M., for 3, 7, and 32 days. Each dose consisted of 0.1 cc of 1:1000 aqueous epinephrine and 0.1 cc of epinephrine-in-oil, 1:500, injected subcutaneously in separate sites. The morning following the last injection of epinephrine (approximately 17 hours later) 2 microcuries of I^{131} was administered intraperitoneally. Twenty-four hours following the administration of the radioactive iodine the animals were killed by the intraperitoneal administration of Nembutal, and the thyroid glands removed.

In the experiments involving the adrenalectomized animals, the rats were bilaterally adrenalectomized after being on the low iodine diet for a period of not less than 3 weeks. At least one week was permitted to elapse following adrenalectomy before the injections of epinephrine were started. The adrenalectomized animals were maintained during the experimental period by the addition of sodium chloride to the drinking water, in isotonic concentration. Epinephrine was administered twice a day for 3 days in the same dosage given to the intact animals. In one experiment, one group of adrenalectomized animals received simultaneous injections of both epinephrine and 17-hydroxy-11 dehydrocorticosterone. The latter was administered subcutaneously in a dosage of 2 mg daily for 3 days.

The radioactive iodine was administered and the animals sacrificed in the same manner described for the intact animals. The thyroid glands were carefully dissected free of connective tissue weighed on a torsion balance, and placed in 2 cc of 1% sodium hydroxide. Digestion was carried out over a steam bath. The solution was diluted to 4 cc, an aliquot removed, placed in a metal cap, and allowed to dry. The β radiation of the sample was measured with a Geiger counter employing the original solution of I^{131} as a standard for calculation of the percentage uptake.

Results. In Table I are presented the results obtained in the intact animals. It will be noted that in each experimental group the percentage uptake of I^{131} is considerably greater in the control group than in the comparable group treated with epinephrine. The percentage uptake of I^{131} of the treated group varied from 45.3 to 66.8% of that of the untreated controls.

The injection of epinephrine (Table II) into the adrenalectomized animals, however, resulted in an increase in the percentage uptake of I^{131} as compared to that of the

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[*] The Steenboch diet is as follows:

Yellow corn	76%
Wheat gluten	20%
Calcium carbonate	3%
Sodium chloride	1%
Irradiated yeast	4 g/k

Drinking water containing 0.06 μg KI/cc

[2] Rawson, R. W., Tannheimer, J. F., and Peacock, W., *Endocrinol.*, 1944, **34**, 245.

[†] The 17-hydroxy-11-dehydrocorticosterone was kindly supplied by the Merck Co., Rahway, N. J.

TABLE I. Normal Rats.

Treatment with epinephrine, days	Epinephrine injected				Controls				% uptake of treated animals as compared to controls %
	No. of rats	Avg body wt, g	Avg thyroid wt, mg	Avg I^{131} uptake, %	No. of rats	Avg body wt, g	Avg thyroid wt, mg	Avg I^{131} uptake, %	
3	5*	140	9.2 ± 0.9§	7.3 ± 1.4	6*	149	10.1 + 1.0	16.1 ± 5.5	45.3
7	6*	162	9.8 ± 1.5	12.4 ± 5.9	5*	187	11.2 ± 1.3	26.7 ± 4.3	48.3
32	6*	147	9.9 ± 2.3	23.7 ± 6.7	6*	162	11.3 ± 1.4	35.5 ± 6.3	66.8
TABLE II. Adrenalectomized Rats.									
3	4*	136	9.5 ± 0.4	23.6 ± 1.8	4*	139	10.4 ± 0.6	16.7 ± 6.4	141
3	3†	153	8.4 ± 1.0	27.6 ± 9.8	4†	157	9.6 ± 1.8	17.7 ± 2.8	156
3	3†	163	14.3 ± 0.7	28.0 ± 6.6	4†	170	12.0 ± 2.0	22.0 ± 4.8	127
17-hydroxy-11-dehydro-corticosterone	3†‡	163	14.1 ± 1.4	17.8 ± 1.0					81

* Wistar strain rats. † Sprague-Dawley rats. ‡ 2 mg daily for 3 days. §S.D.

non-injected adrenalectomized controls. In these experiments the percentage uptake of the administered I^{131} in the epinephrine treated adrenalectomized rats varied from 127 to 156% of that of the untreated adrenalectomized rats. In one small group of adrenalectomized animals injected with both epinephrine and 17-hydroxy-11-dehydrocorticosterone, there occurred a reversal of this phenomenon with a marked reduction in the uptake of radioactive iodine.

Conclusions. The injection of epinephrine into the intact rat results in a considerable decrease in the uptake of I^{131}. This is in sharp contrast to the results obtained in similarly treated adrenalectomized rats. In this group, the injections of epinephrine after adrenalectomy is followed by a pronounced increase in the percentage uptake of radioactive iodine. These experiments would indicate that the adrenal cortex exercises an inhibitory effect on the thyrotropin stimulating action of epinephrine. The results obtained in the rat are somewhat different from those observed in the dog, in that in the latter animal the inhibitory effects of the adrenal are not manifest until after several days of treatment with epinephrine. This apparently represents a species difference, although a different technique was employed.

In one preliminary group of experiments it was found that the parenteral administration of 17-hydroxy-11-dehydrocorticosterone, one of the carbohydrate acting fractions of the adrenal cortex, inhibited the thyrotropin stimulating effect of epinephrine.

That the decrease in uptake of I^{131} in the epinephrine treated intact animals is not due to the vasoconstrictor effects of the epinephrine is evidenced by the fact that in similarly treated adrenalectomized animals the percentage uptake of I^{131} is considerably greater than that of the untreated controls. A period of 17 hours after the last injection of epinephrine was allowed to elapse before the administration of I^{131} in order to avoid any possible vasoconstrictor effects.

We wish to extend our sincere thanks to the Physics Laboratory of the Hospital for their aid with the isotope studies.

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17103. Some Chemical and Morphological Changes Elicited in the Adrenal by Stilbestrol and LAP.

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Preliminary observations suggested that a lyophilized anterior pituitary preparation (LAP), when administered simultaneously with stilbestrol, inhibits the discharge of adrenal sudanophilic material which is normally a characteristic effect of this synthetic estrogen. Hence the following experiment was planned to study in a more comprehensive manner the chemical and morphological changes produced in the adrenal by LAP, stilbestrol and combined treatment with both these substances.

Materials and methods. 48 female piebald rats averaging 140 g in weight were divided into 4 groups of 12 rats each and treated as follows: Group I served as normal controls. Group II received 20 mg of LAP in 0.2 cc of saline twice daily subcutaneously. Group III was given 1 mg of stilbestrol dissolved in 0.2 cc of corn oil once daily subcutaneously. In addition this group received a saline injection on the 9th day (to equalize the injection-trauma with Group IV). Group IV was given both LAP and stilbestrol in the same manner as Groups II and III respectively. At the end of 10 days the rats were killed by ether anesthesia. The adrenal glands from 6 rats of each group were immediately removed and weighed separately to 1 mg of accuracy. One gland of each rat was then placed into 4% trichloracetic acid for ascorbic acid determination (method of Roe and Kuether[1]) and the other into acetone-alcohol for cholesterol estimation (method of Sperry and Schoenheimer, modification of Sperry[2]).

The adrenals of the other 6 rats of each group were used for histological study as follows: one was fixed in 10% formalin for 48 hours after which frozen sections were made at 10 μ. These were stained for lipids with Sudan IV and for "Plasmalogens" with the Schiff reagent (method of Feulgen and Voigt as described by Lison[3]). The other adrenal and thymus were fixed in Heidenhain's Suza fixative for H and E paraffin sections cut at 6 μ.

Results. A summary of our results is given in Table I which includes a statistical comparison of the values obtained.

Weight changes. As thymus and adrenal weight changes are inversely related to each other, they can be considered together. In all experimental groups there was adrenal enlargement and thymus involution. These changes were most pronounced in Group IV and least marked in Group II.

Chemical changes. The cholesterol and ascorbic acid values are expressed in mg/100 mg of adrenal tissue (concentration) and in mg/100 g of body weight (total content).

After LAP treatment (Group II) there is a decrease in the concentration and total content of adrenal *cholesterol;* only the decrease in concentration being significant. Stilbestrol (Group III) causes a very marked and significant decrease in both concentration and content of adrenal cholesterol. After LAP and stilbestrol (Group IV) these values were still lower, but not significantly different from the former group (Group III).

In a progressive order, LAP (Group II), stilbestrol alone (Group III) and in conjunction with LAP (Group IV) significantly decreased the adrenal *ascorbic acid* concentration. It is of interest that while the changes in the total content and concentration of cholesterol run parallel, no such parallelism is evident as regards ascorbic acid.[4]

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* This work was initiated while the author was a holder of a research fellowship from the Hoffman-LaRoche Co., Nutley, N. J.

† This work was done during the tenure of a Life Insurance Medical Research Fellowship.

[1] Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 1943, **147**, 399.

[2] Sperry, W. M., *Am. J. Clin. Path.*, 1938, **2**, 91.

[3] Lison, L., Histochimie Animale, Gauthier-Villars, Paris, 1936.

[4] Ludewig, S., and Chanutin, A., *Endocrinology*, 1947, **41**, 135.

TABLE I.
Effect of LAP* and Stilbestrol on Thymus and Adrenal Weight and Adrenal Cholesterol and Ascorbic Acid.

		Thymus weight		Adrenal weight	
Group	Treatment	Mg/100 g body	P†	Mg/100 g body	P
I	0	116 ± 22		32 ± 2.5	
II	LAP	47 ± 6.5	II-I <.01	36 ± 0.5	II-I <.01
III	Stilbestrol	56 ± 4.8	III-I <.02	79 ± 6.6	III-I <.01
IV	Stilbestrol LAP	21.5 ± 1.5	IV-I <.01	85.9 ± 14.5	IV-I <.01
			IV-II <.01		IV-II <.01
			IV-III <.01		IV-III >.1

		Adrenal cholesterol			
Group	Treatment	Mg/100 g body	P	Mg/100 mg adrenal tissue	P
I	0	.623 ± .088		2.674 ± .168	
II	LAP	.375 ± .076	II-I >.1‡	1.047 ± .231	II-I <.01
III	Stilbestrol	.300 ± .058	III-I <.02	.385 ± .087	III-I <.01
IV	Stilbestrol LAP	.186 ± .021	IV-I <.01	.227 ± 0.15	IV-I <.01
			IV-II <.05		IV-II <.01
			IV-III <.1‡		IV-III >.1

		Adrenal ascorbic acid			
Group	Treatment	Mg/100 g body	P	Mg/100 mg adrenal tissue	P
I	0	.105 ± .014		.442 ± .050	
II	LAP	.117 ± .008	II-I >.1‡	.322 ± .020	II-I <.05
III	Stilbestrol	.168 ± .027	III-I <.1‡	.222 ± .050	III-I <.02
IV	Stilbestrol LAP	.097 ± .014	IV-I >.1‡	.122 ± .021	IV-I <.01
			IV-II >.1‡		IV-II <.01
			IV-III <.05		IV-III >.1‡

* Lyophilized Anterior Pituitary.

† Probability of a chance occurrence of the difference between the means, from Fisher's table of T values.

‡ Difference not statistically significant.

Histology. As the results of Sudan and Schiff stains were in the same direction, only the former will be discussed.

The sudanophilia of the *adrenal* cortex was greatly increased by LAP. In contrast stilbestrol caused a marked discharge of sudanophilic granules. Curiously, combined stilbestrol and LAP treatment actually increased the cortical lipid content above normal, with a maximal concentration in the outer fasciculata.

Examination of H. and E. sections revealed a predominant hyperplasia of the adrenals with slight hyperemia in the group treated with LAP. The stilbestrol and especially the LAP and stilbestrol treated rats evidenced a degree of hyperplasia which was completely overshadowed by a marked congestion and edematous imbibition resulting in severe disruption of the glandular structure.

In the *thymus* there was nuclear pyknosis which appeared to parallel the degree of atrophy.

Discussion. Hypertrophy,[5,6] loss of sudanophilic material[7-9] as well as depletion of cholesterol[10-12] and ascorbic acid[13-15] followed by

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[5] Selye, H., *Brit. J. Exp. Pathol.*, 1936, **17**, 234.

[6] Tepperman, J., Engel, F. L., and Long, C. N. H., *Endocrinology*, 1943, **32**, 373.

[7] Dalton, A. J., Dosne, C., and Selye, H., *Anat. Rec.*, 1940, *Suppl.* **76**, 85.

[8] Zwemer, R. L., *Am. J. Pathol.*, 1936, **12**, 107.

[9] Darrow, D. C., and Sarason, E. L., *J. Clin. Invest.*, 1944, **23**, 11.

[10] Popjack, G., *J. Path. and Bact.*, 1944, **56**, 485.

[11] Levin, L., *Endocrinology*, 1945, **37**, 34.

[12] Long, C. N. H., *Rec. Progr. in Horm. Res. Proc. Laur. Horm. Conf.*, 1947, **1**, 99.

a return of these substances to or above normal levels, have been repeatedly described as characteristic of the adrenal response to stress. The alterations in cholesterol, ascorbic acid and stainable lipids have been considered by Sayers and Sayers[16] to closely parallel each other. Indeed the observation by these workers of a simultaneous decrease in cholesterol and sudanophilic material has prompted them to identify the latter with cholesterol esters. The present study shows that it is possible to dissociate the sudanophilia from the cholesterol content of the adrenal cortex. LAP, either alone or in conjunction with stilbestrol, decreases the cholesterol but increases the sudanophilia of the adrenal. This suggests that the sudanophilic substance of the cortex is probably not cholesterol. Earlier experiments[17] have demonstrated that, under certain conditions, purified ACTH can cause a similar dissociation. Finally it appears from the above data that LAP, given with stilbestrol, markedly inhibits that loss of sudanophilic granules from the cortex which is characteristic of stilbestrol treatment.

Further experiments are under way to study the effects of purified corticotrophic preparations on the adrenal changes elicited by non-specific stress.

Summary. The adrenal cholesterol and ascorbic acid levels were studied in relation to the stainable lipids of the gland in rats treated with a lyophilized anterior pituitary preparation (LAP), given alone or in conjunction with a synthetic folliculoid (stilbestrol):

1. In stilbestrol treated rats, an almost total depletion of stainable lipids was observed in conjunction with low cholesterol and ascorbic acid values.

2. LAP caused accumulation of sudanophilic lipids in the adrenal cortex but significantly lowered the cholesterol and ascorbic acid levels.

3. A similar dissociation of sudanophilic material from cholesterol and ascorbic acid, was even more evident when LAP was administered concurrently with stilbestrol. The effect of stilbestrol on sudanophilia was counteracted by LAP.

4. These observations show that the corticotrophic effect of folliculoids cannot be solely ascribed to the liberation of anterior pituitary corticotrophin such as is present in LAP.

This investigation was supported by a research grant from the National Heart Institute, U. S. Public Health Service. The authors gratefully acknowledge the histological assistance of Dr. P. S. Timiras and the technical help of K. Nielsen and M. Parent.

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[13] Torrance, C. C., *J. Biol. Chem.*, 1940, **132**, 575.

[14] Harkins, H. N., and Long, C. N. H., *Am. J. Physiol.*, 1945, **144**, 661.

[15] Sayers, G., and Sayers, M. A., Tsan-Ying Liang, and Long, C. N. H., *Endocrinology*, 1945, **37**, 96.

[16] Sayers, G., and Sayers, M. A., *Rec. Progr. in Horm. Res., Proc. Laur. Horm. Conf.*, 1948, **2**, 81.

[17] Constantinides, P., Fortier, C., Skelton, F. R., Timiras, P., and Selye, H., in press.

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17104. Effect of Various Drugs on Epinephrine-Induced Pulmonary Edema in Rabbits.*

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Large doses of epinephrine are known to cause a fulminating pulmonary edema in a number of species of animals.[1-3] Various

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* Parke, Davis and Co., Detroit, Mich., supplied a grant in support of this investigation, as well as the adrenergic blocking drugs employed.

[1] Meltzer, S. J., *Am. Med.*, 1904, **8**, 191.

[2] Emerson, H., *Arch. Int. Med.*, 1909, **3**, 368.

[3] Bovet, D., and Bovet-Nitti, F., *Structure et activité pharmacodynamique des médicaments du système nerveux végétatif*, S. Karger, Bale, 1948, p. 23.

workers have claimed that atropine,[4,5] hypnotics,[6] tissue extracts,[7] antihistaminics[8] and pituitrin[9] can diminish or prevent this edema formation. Furthermore, various adrenergic blocking drugs, as well as vasodilator drugs have been shown to be capable of reducing the mortality which follows toxic doses of epinephrine in mice[10] and rats.[11,12] A variety of explanations concerning the cause of pulmonary edema have been based, in part, on data relating to the effects of drugs on the toxicity of epinephrine.

The drugs which have been reported to protect against epinephrine-induced pulmonary edema vary widely in properties and several are relatively specific in action. If the claims made are true it would suggest that numerous mechanisms are involved in edema production or that the several protective drugs have a common property, which seems quite unlikely. Preliminary to studies aimed more directly at the elucidation of the mechanisms involved in the production of pulmonary edema and the control by drugs it was considered advisable to re-examine the effects of some of these drugs on epinephrine-induced pulmonary edema under standardized experimental conditions.

Method. Drugs were administered as indicated in Table I to male rabbits weighing between 2 and 3 kg prior to the intravenous injection, in 15 seconds, of 0.375 mg/kg of epinephrine (calculated as the base). The epinephrine solution employed (0.1%) was

TABLE I. Alteration of Pulmonary Edema and Mortality Due to Large Doses of Epinephrine in Rabbits.

	No. exp.	Mean body wt, kg	Mean lung wt, g ± s	Diff. from epinephrine controls, g ± s	P*	% mortality†	Diff. from epinephrine controls	P*
			Control Groups.					
Epinephrine	18	2.32	21.77 ± 7.19	—	—	61.1	—	—
Normal lung wt	12	2.59	10.58 ± 1.41	—	—	—	—	—
			Pretreated Groups‡					
Pentobarbital Na, 25 mg/kg I.V.	6	2.52	22.34 ± 6.36	+ 0.57 ± 3.3	>0.5	66	+ 4.9	>0.5
Phenobarbital " 50 " S.C.	6	2.60	34.95 ± 14.49	+13.18 ± 4.31	<0.01	80.8	+28.9	>0.1, <0.2
Neoantergan maleate, 2.5 mg/kg I.V.	9	2.33	19.44 ± 5.99	— 2.33 ± 2.80	>0.4	55.6	— 5.5	>0.5
Phenergan HCl, 1.5 mg/kg I.V.	7	2.74	26.21 ± 10.84	+ 4.44 ± 3.59	>0.2	57.1	— 4.0	>0.5
" " 20 " S.C.	8	2.69	24.53 ± 6.05	+ 2.76 ± 2.90	>0.3	12.5	—48.6	<0.01
Atropine sulfate, 5 mg/kg I.V.	9	2.37	14.95 ± 6.31	— 6.83 ± 2.44	>0.02, <0.05	33.0	—28.1	>0.1, <0.2
SY-28,§ 0.25 mg/kg I.V.	5	2.40	9.13 ± 0.66	—12.64 ± 3.27	<0.01	0	—61.1	<0.01
SY-2,‖ 2.5 mg/kg I.V.	6	2.36	10.83 ± 2.31	—10.94 ± 3.02	<0.01	0	—61.1	<0.01

* Statistically significant when 0.05 or less.

† Mortality figures are those based on the numbers of animals dying spontaneously during 15-minute observation period following epinephrine.

‡ Intravenous pretreatment was instituted 10 minutes before epinephrine. Drugs administered subcutaneously were given 30 minutes before the standard intravenous dose of epinephrine of 0.375 mg/kg (as the base).

§ N-(2-bromoethyl)-N-ethyl-1-naphthalenemethylamine • HBr. ‖ N-(2-chloroethyl)-N-ethylbenzhydrylamine • HCl.

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[4] Auer, J., and Gates, F. L., *J. Exp. Med.*, 1917, **26**, 201.

[5] Bariety, M., and Kohler, D., *Compt. rend. Soc. de biol.*, 1948, **142**, 280.

[6] Luisada, A., *Arch. f. Exp. Path.*, 1928, **132**, 313.

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[9] Elkeles, A., *Brit. J. Radiol.*, 1948, **21**, 472.

[10] Loew, E. R., and Micetich, A., *J. Pharm. and Exp. Therap.*, 1948, **93**, 434.

[11] Nickerson, M., and Goodman, L. S., *J. Pharm. and Exp. Therap.*, 1947, **89**, 167.

[12] Rothlin, E., *Bull. de l'Acad. Suisse des Sci. Med.*, 1946-47, **2**, 1.

made by dissolving the base in saline with the addition of a few drops of HCl. Concentrations of other drugs in saline were adjusted so that a given dose was administered in 0.5 ml/kg. Control data were obtained concurrently from animals receiving only epinephrine or no treatment whatsoever. Following the intravenous injection of epinephrine, the rabbits were observed for 15 minutes, at which time, if surviving, they were sacrificed by a sharp blow on the head. All lungs were removed 10 minutes after death, freed of adhering tissue, blotted on absorbent paper and wet-weighed. The indication of effectively altering the toxic response to epinephrine was the presence of a statistically significant reduction in lung weight and/or mortality from that of the controls receiving only epinephrine (Table I).

Results and discussion. The lungs of epinephrine-treated rabbits were markedly hemorrhagic and edematous and weighed double that of normal lungs. A frothy fluid, usually uncolored but sometimes salmon colored, frequently completely filled the trachea and bronchi and appeared to account for the respiratory embarrassment, cyanosis and asphyxial convulsions which preceded death. Fluid frequently escaped from the nostrils and mouth during asphyxial convulsions or when survivors were killed 15 minutes after injection of epinephrine. This fulminating, severe pulmonary edema was probably the main cause of death which occurred in 3 to 15 minutes in 61% of the 18 control animals. Pretreatment with a variety of drugs was instituted in attempts to prevent the edema. During each daily experiment several control rabbits and several rabbits treated with each drug were included, thus yielding data which could be pooled and compared (Table I).

Barbiturates. Luisada[6] reported that hypnotics in general were capable of reducing the mortality and pulmonary edema induced with epinephrine in rabbits. He believed that such drugs protected by suppressing a central component of a contributory pulmonary vascular reflex which was brought about by epinephrine or the resultant severe hypertension.[6] The results herein reported with pentobarbital in a near anesthetic dose and phenobarbital in a sedative dose do not support such a contention. Neither of these barbiturates exerted any protective action under conditions which appear to be essentially identical to those described by Luisada. Phenobarbital actually caused a significant increase in lung weight and the data suggest an increased mortality.

Antihistaminics. Experiments conducted by Staub[13] indicate that epinephrine may cause the release of significant amounts of histamine in the body. If the toxic effects of large doses of epinephrine in rabbits are accentuated by histamine released from tissues, it would be expected that pretreatment with antihistaminics would confer protection.

The treatment with Pyranisamine (Neoantergan maleate[†]), a potent and specific antihistaminic did not prevent the increase of lung weight or reduce mortality due to epinephrine (Table I). N-β-Dimethylaminopropylthiodiphenylamine.HCl (Phenergan, 3277 RP)[†] administered intravenously likewise afforded no protection, although larger doses injected subcutaneously reduced mortality but did not decrease pulmonary edema. This suggests that death in rabbits due to epinephrine is not attributable solely to pulmonary edema, or that undetected decreases in degree of edema were sufficient to reduce mortality.

These data do not confirm the results reported by Halpern *et al.*[8] who found that the same large doses of Phenergan under essentially the same experimental conditions completely protected rabbits from the toxic effects of epinephrine. Reuse[14] using the same doses of Phenergan in guinea pigs, reported that this drug reduced mortality due to epinephrine, although the protection conferred by Phenergan was not complete. He states that immediately after epinephrine, the treated animals showed signs of respiratory embarrassment. While this suggests the presence of congestion and edema, no routine examination of the lungs was made. However, the lung weights of 3 such animals were somewhat above normal, even at 2 hours after epinephrine.

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[13] Staub, H., *Experientia*, 1946, **2**, 29.

[†] Supplied through the courtesy of Merck and Co., Rahway, N. J.

[14] Reuse, J. J., *Compt. rend. Soc. de biol.*, 1948, **142**, 638.

Atropine. Various authors[4,5] have claimed that atropine protects against epinephrine-induced pulmonary edema. Phenolic ethers of amino alcohols, possessing atropine-like properties as well as antihistamine and epinephrine blocking properties, are said to protect.[5] On the other hand, significant protection by atropine has been denied.[6,15]

Table I shows that a large intravenous injection of atropine sulfate partially prevented to a significant degree the increase of lung weight. Mortality was not significantly decreased probably because of the small number of animals employed. However, doses less than 5.0 mg/kg did not protect, and the dose necessary to confer protection was relatively large considering the potency of the agent. In addition, the protective effect of atropine is slight when compared to that produced by the adrenergic blocking agents.

Adrenergic blocking drugs. It has been shown that N-(2-bromoethyl)-N-ethyl-l-naphthalenemethylamine.HBr (SY-28) is a potent adrenergic blocking agent,[16,17] as well as one of the most potent histamine antagonists known.[16] A closely related compound, N-(2-chloroethyl)-N-ethylbenzhydrylamine.HCl (SY-2) is a specific adrenergic blocking agent.[18] Both of these compounds, in comparatively small doses, prevented death and pulmonary edema due to epinephrine in rabbits, as is shown in Table I. It is emphasized that these were the only drugs studied which completely protected the animals against the toxic effects of epinephrine.

Other adrenergic blocking agents are capable of reducing epinephrine-induced pulmonary edema. Rothlin[15] recently reported that ergotamine had such properties. Dibenamine and various other derivatives of β-chloroethylamine, benzyl-imidazoline (Priscol) and yohimbine, when given orally, reduced the toxicity of epinephrine injected intraperitoneally in mice.[10] Under these conditions, the dioxanes, 883F and 933F, were ineffective following moderate doses in mice.[10] Presumably the protective action of most adrenergic blocking drugs in mice is due to the prevention of pulmonary edema since increased lung weights were readily demonstrable after epinephrine injection.[19]

We believe the data obtained from rabbits and herein presented and the findings reported for mice,[10] rats[11,12,15] and rabbits,[6] reveal clearly that pulmonary edema and mortality following epinephrine administration are most effectively reduced by vasodilator drugs which are physiological antagonists of epinephrine and by adrenergic blocking drugs which pharmacologically block excitatory actions of epinephrine such as vasoconstriction and the resultant hypertension.

A more direct examination of the acute hypertensive action of epinephrine, and of the pressures within pulmonary vessels and of the effect of drugs thereon would appear to be the most promising approach to elucidation of factors involved in epinephrine-induced pulmonary edema. Hamilton, Woodbury, and Vogt[20] recorded pressure changes in the pulmonary artery and vein of the unanesthetized dog after large, but non-fatal, doses of epinephrine and observed a rise of pressure in both. They concluded that the increased pressures were due to failure of the left ventricle as a result of the high arterial pressure. Furthermore, direct observations of the heart by Auer and Gates,[4] Johnson,[21] and Barach, *et al.*[22] indicate that the left ventricle fails in the rabbit as well. This suggests that a high pulmonary circulatory pressure prevails after toxic doses of epinephrine and could account for the rapid transudation of fluid into the lungs.

Summary. 1. Pulmonary edema and mortality induced in rabbits by intravenous injections of epinephrine were completely prevented by comparatively small doses of adrenergic blocking drugs, N-(2-bromoethyl)-N-ethyl-l-naphthalenemethylamine.HBr (SY-28) and N-(2-chloroethyl) - N - ethylbenzhydrylamine.HCl (SY-2).

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2. It would appear unlikely that epinephrine toxicity and the pulmonary edema involved are importantly related to histamine release from tissues since the antihistaminics, Pyranisamine (Neoantergan maleate) and Phenergan (3277 RP), failed to prevent edema although large subcutaneous doses of Phenergan reduced mortality.

3. The barbiturates, pentobarbital sodium and phenobarbital sodium did not decrease mortality or edema.

4. The available evidence indicates that epinephrine toxicity is most readily reduced by vasodilator drugs which represent physiological antagonists, and by adrenergic blocking drugs which are pharmacological antagonists of epinephrine.

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17105. Myelination of the Hypothalamus and Its Relation to Thermoregulation in the Hamster.*

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Correlation of the ability to regulate body temperature in a cold environment with progressive myelination of the hypothalamus has been previously demonstrated in albino rats.[1] The ability of rats to maintain body temperature is acquired at ages ranging between 18 and 30 days. The earlier literature indicating that function of fiber tracts in the central nervous system may be dependent upon myelination of their component nerve fibers was briefly reviewed in a previous report.[1]

In the present report the results of an investigation of myelination in the hypothalamus of the hamster and the development of its ability to regulate against cold are presented. The investigation seemed indicated in view of the fact that hamsters are more immature at birth than rats.

Methods and materials. Six litters of Syrian hamsters were used in the present study. Their body temperatures were recorded by means of a Brown electronic continuous recording potentiometer and copper-constantan thermopiles. The thermopiles were inserted into the colons of the animals to a depth of 4-5 cm. Animals varying from 6-71 days of age were subjected to environmental temperatures of 5-8°C for periods varying from 30 minutes to 20 hours. After exposure the hamsters were anesthetized and perfused with 3½% potassium dichromate followed by Regaud's solution. The brains were then removed, divided in the median sagittal plane and placed in 5% potassium dichromate where they remained for 10 days days at a temperature of 37-39°C. After washing, dehydration, embedding, and sectioning at 20 μ they were stained according to the method of Ulett, Dow, and Larsell.[2] Sections were made in the parasagittal plane to permit study of all areas of the hypothalamus (rostral, caudal and middle) in each section. Representative sections from the lateral hypothalami of all members of a given litter were stained en masse and myelin was quantitated by means of a Photovolt electronic photometer as previously described.[1] In only two instances, because of the difficulty in procuring large litters of hamsters, was a litter-mate control sacrificed with the experimental animals.

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* Supported by a grant from the Office of Naval Research.

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Results. The first animal from the first litter of hamsters studied was subjected to cold stress at 6 days of age and the other members were successively stressed on the 13th, 18th, 20th, 25th, and 32nd days. The duration of cold stress was 30 minutes. The fall in body temperature of the first animal was approximately 20°C. The older hamsters gradually improved in ability to regulate until the 32nd day when the temperature of the last animal in the litter fell only 3°C. The concentration of myelin in the dorsolateral areas of the caudal hypothalami, as determined photometrically, increased with age and reached its highest level in the 32-day animal. A parallel increase was evident in the myelin of the medullary pyramids but the concentration was considerably higher than in the hypothalamus at all ages.

The members of the second litter were cold-stressed for 30 minutes at ages ranging from 12-36 days. Regulation was best in the 31-day animal in which hypothalamic myelin was also at its highest level. The same was true of Litter No. 3 which was cold-stressed (30 minutes) at ages ranging from 18-46 days. Deterioration in regulatory ability and some apparent decrease in myelin were observed in the 3 members of this litter over 31 days of age (Fig. 1).

Cold stress was continued for 2 hours in the fourth litter studied (Fig. 2). Body temperatures of these hamsters averaged approximately 1°C lower at the end of 2 hours in the cold room than at 30 minutes. Myelin reached its highest level in the 25- and 30-day animals and regulatory ability was best at 30 days. Myelin appeared to decrease in hamsters over 30 days of age and there was an associated deterioration of regulatory ability which was most evident in the 55-day animal after 2 hours of exposure.

There were 10 hamsters in the fifth litter studied which may account for the fact that attainment of regulatory ability was somewhat delayed. Regulation was best at 42 days with myelin content of the caudal hypothalami increasing rather rapidly from 18-42 days. Regulation in the 50-, 60-, and 71-day animals was only slightly less efficient than in the younger animals and was actually better after 8 hours of exposure to the cold environment than after 2 hours. Myelin concentration in the hypothalami of the 3 oldest members fluctuated between levels slightly above and slightly below that of the 42-day animal.

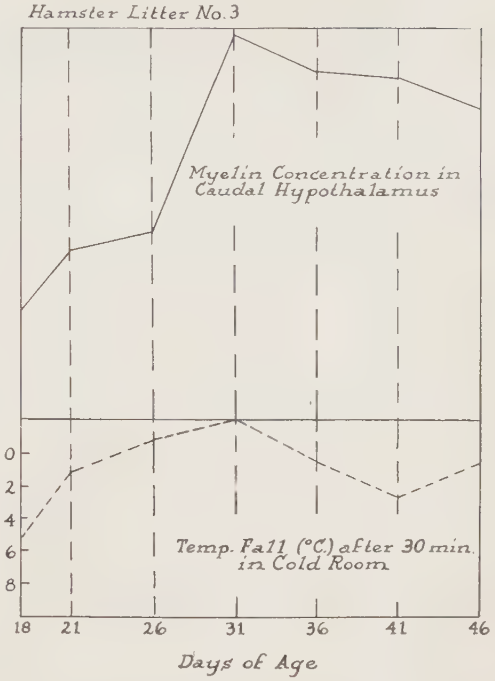

FIG. 1.

Temperature falls after 30 min. at 5-8°C and myelin concentrations in the caudal hypothalami of hamsters in Litter No. 3.

The sixth and last litter also consisted of 10 hamsters, the first of which (18 days) was not included in Fig. 3 since its temperature, after 2 hours of exposure, had dropped below the range of available recording equipment. Regulation against cold was best at 44 days at which time myelin in the caudal hypothalamus was also at its highest level. Litter-mate controls were sacrificed with the 40- and 59-day stressed animals of this litter and, in both instances, showed higher levels of myelin in their caudal hypothalami than the stressed hamsters (Fig. 3). The 40-, 44-, 59-, and 69-day animals were left in the cold room for 9, 11, 17, and 20 hours respectively; all maintained their body temperatures at levels equal to or higher than those manifested after exposure for 2 hours.

Discussion. The experimental data which

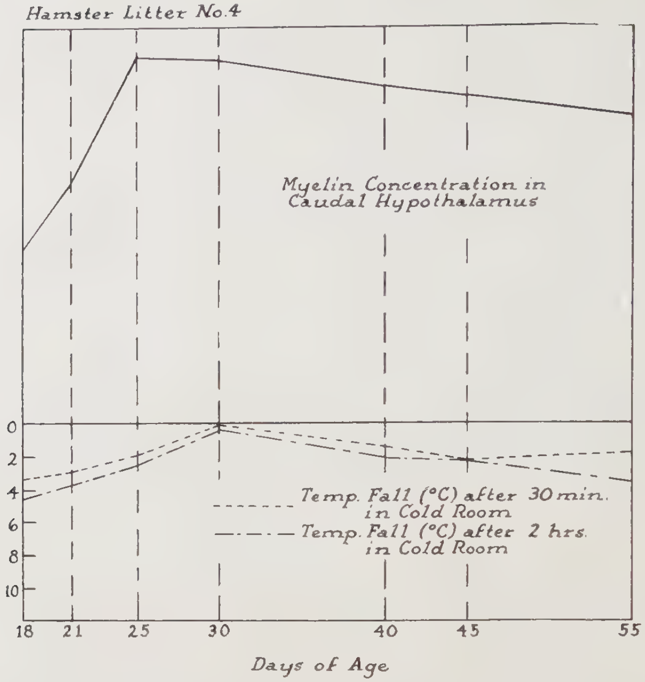

FIG. 2.
Temperature falls after 30 min. and 2 hr in the cold environment and myelin concentrations in the caudal hypothalami of hamsters in Litter No. 4.

have been obtained from subjecting hamsters to an environmental temperature of 5-8°C indicate that their attainment of ability to regulate against cold is considerably more delayed than in albino rats, or 32-44 days of age as compared to 18-30 days. This was to be expected since the gestation period in hamsters is considerably shorter than in rats with the result that hamsters are more immature at birth.

The maintenance of body temperature by reasonably mature hamsters in an environment ranging between 5° and 8°C for periods of 20 hours or more is not surprising. Sexually mature hamsters over three months of age were kept in an environmental temperature of 4° ± 2°C for periods of one or two months by Lyman[3] without appreciable reduction from normal of their body temperatures until they entered hibernation. Most of his hamsters "entered hibernation within a month or two after being brought into the cold room"; during hibernation their temperatures were only about 1°C above that of the environment.

As is the case in rats,[1] myelination, determined photometrically, reaches its highest level in the caudal hypothalamus of the hamster at the age when regulatory function is established. This observation, in an additional rodent, supports the hypothesis previously advanced that the role of the hypothalamus in thermoregulation is facilitated, at least in part, by myelination of the axons of hypothalamic neurons.

The observation that myelin concentration in the hypothalamus is higher, according to our method of measurement, in unstressed than in stressed animals from the same litter and at the same age suggests mobilization or change in chemical composition of myelin as a part of the metabolic reaction to cold stress. This may be a more prominent feature in older animals in which the process of primary myelination is essentially complete and it may account for the apparent tendency for myelin

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[3] Lyman, C. P., *J. Exp. Zool.*, 1948, **109**, 55.

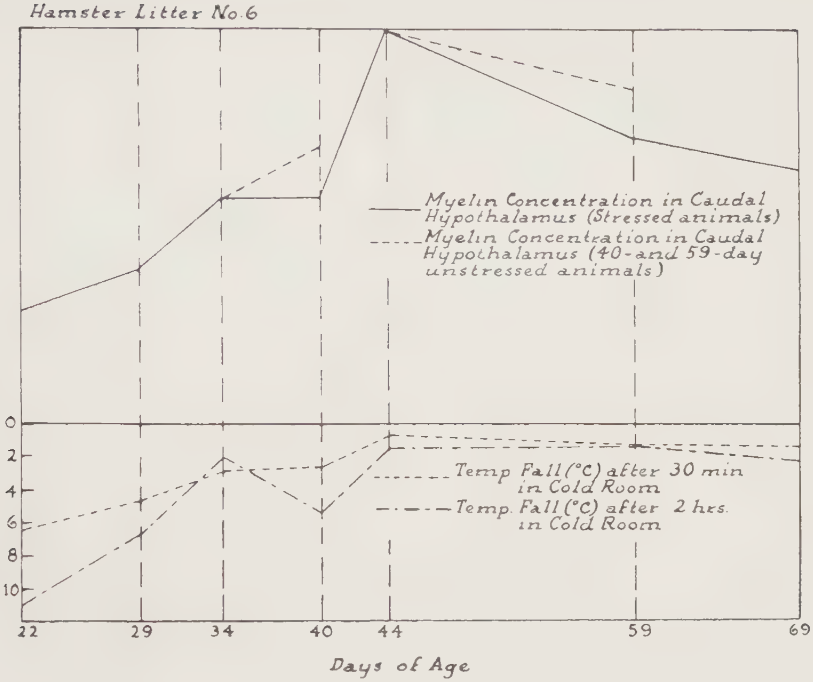

FIG. 3.

Temperature falls after 30 min. and 2 hr in the cold environment and myelin concentrations in the caudal hypothalami of hamsters in Litter No. 6. The differences between myelin concentrations in stressed and unstressed animals at 40 and 59 days of age are also shown.

to decrease after reaching its highest level in hamsters and in the rats previously studied.[1] All the animals heretofore used in plotting myelin curves have been stressed animals, sacrificed immediately after removal from the cold environment. It is unlikely that the mild but consistent deterioration in regulatory ability evident in the older animals (Fig. 2 and 3) is directly related to the decrease in density of stained myelin in the hypothalami, but the possibility cannot be overlooked.

Summary. Hamsters acquire the ability to effectively regulate their body temperatures in a cold environment (5° - 8°C) at ages ranging from 30-44 days. Myelination in the dorsolateral areas of the caudal hypothalamus, as determined photometrically, reaches its height at the time thermoregulation becomes fully developed. It appears therefore that myelination of hypothalamic neurons may be essential to functional maturation of the hypothalamus. The data with respect to hamsters are similar and lend support to those previously reported for albino rats.

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17106. Effect of BAL on Experimental Polyarthritis of Rats.*

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BAL (2:3-dimercaptopropanol) is used in counteracting gold toxicity which appears occasionally during chrysotherapy of rheumatoid arthritis in man. Experimental polyarthritis of rats produced by the L-4 strain of a pleuropneumonia-like organism likewise responds to gold therapy. Although the experimental disease in rats is pathologically not the same as rheumatoid arthritis in man it has been used as a means of making chemotherapeutic trials.[1]

The use of BAL in arthritic patients who are being treated at the same time with gold raises the possibility that the arthritis may be aggravated because the BAL would increase the excretion of gold and so decrease the amount of gold available for the disease process.[2,3] Since gold has a beneficial action in arthritis the use of a substance capable of facile combining with it might *per se* aggravate the arthritis. To examine this possibility the effect of the administration of BAL on the experimental arthritis of rats, not treated with gold, was studied.

Procedure. A total of 41 male and 49 female rats were given a total cumulative dose of 90 to 100 mg per kg body weight of BAL intramuscularly during a period of 6 weeks. For the first 5 days of the experiment 5 mg per kg of BAL (diluted in olive oil) was administered twice daily. Thereafter, the same dose was given twice weekly for 5 weeks.

On the second day of BAL injection the rats received 2.0 cc of a broth culture of the L-4 strain of pleuropneumonia-like organism intraperitoneally. The organisms were cultured according to a method described by Tripi and Kuzell.[1] Evaluation of the arthritic involvement was made by using a modification of the arthrogram described by Sabin and Warren.[4] This consists of a scoring square divided into 4 quarters. Each quarter represents one leg of the rat. Arthritis is noted by blacking out various portions of the square. A means of scoring these arthrograms according to the degree and extent of rat polyarthritis has been described elsewhere.[5] The arthrogram score is based on a possible score of 4 for a front leg and 5 for a hind leg, giving a maximum score of 18 for the animal.

A total of 37 male and 43 female rats was used as controls and received only the pleuropneumonia-like organism intraperitoneally. Ten rats injected with olive oil alone in comparable dosage showed no difference from these controls. There was no demonstrable change in growth of pleuropneumonia-like organisms in broth cultures *in vitro* to which pure BAL[†] had been added in concentrations of from 0.0007 to 0.2 mg per cc. Infected broth cultures containing BAL in a concentration of 0.2 mg per cc were injected intraperitoneally into rats and the typical arthritis was produced. Increasing the concentration of BAL in the broth cultures beyond 0.2 mg per cc inhibited the growth of the microorganism. Five rats (average weight 60 g) were given 5 mg per kg of BAL intramuscularly twice daily for 5 days and thereafter twice weekly for 5 weeks. These rats were compared with similar untreated controls regarding weight gain. There was no demonstrable dif-

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* This work was done in part under contract with the Office of Naval Research, U. S. Navy Department, and supported, in part, by the Stern Fund for Experimental Arthritis.

[1] Tripi, H. B., and Kuzell, W. C., *Stanford Med. Bull.*, 1947, **5**, 98.

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[3] Kuzell, W. C., Pillsbury, P. L., and Gellert, S. A., *Stanford Med. Bull.*, 1947, **5**, 197.

[4] Sabin, A. B., and Warren, J., *J. Bact.*, 1940, **40**, 823.

[5] Tripi, H. B., Gardner, G. M., and Kuzell, W. C., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 45.

[†] Supplied by Hynson, Westcott and Dunning, Inc., Baltimore, Md.

TABLE I.
Effect of BAL on Incidence and Extent of Arthritis and on Survival Rate of Infected Animals.

	Arthritis		
	Incidence, %	Extent (Arthrogram Avg)	Survival, %
BAL + pleuropneumonia infection	73	2.86	89
Pleuropneumonia infection only	55	1.83	95

The standard error of difference in incidence of arthritis is 7.3% while the observed difference is 18% so that the chance occurrence of the observed difference is 1:80.

ference in weight between these groups, thus indicating that the BAL had little general toxic effect on the rats.

Results. The arthrogram scores given in Table I compare the average arthritic involvement in the group receiving both BAL and the infection with that receiving the infection only. The extent of the average arthritic involvement in the individual animals receiving BAL is greater than in the infected controls. The survival rate is slightly less in the group receiving BAL. The incidence of arthritis is greater in the BAL medicated group.

Discussion. These results indicate that the administration of BAL increases the severity of an experimental arthritic process. Such a difference might be attributable to the general toxic effects of BAL rather than to direct enhancement of the growth of the infecting organism, but our failure to show a difference in weight between animals given BAL alone and untreated controls argues against this possibility. Peters *et al.* found that LD_{50} from the subcutaneous administration of BAL in oil to rats was 110 mg per kg body weight.[6] Waters and Stock[7] reported the American Reference Standard LD_{50} to be 105 mg per kg intramuscularly in rats. It would, therefore, appear that single doses of 5 mg per kg would not be expected to produce appreciable toxicity. The question then arises as to whether the slight toxic effects produced by low doses could increase the severity of the arthritis. This does not seem likely, since it has been found that other toxic substances such as 0.1% sodium phenobarbital, 0.01% arsenic trioxide, or 0.1% isopropyl alcohol in the drinking water for prolonged periods caused no increase in the severity of this experimental polyarthritis of rats.[8]

It is possible that BAL in low concentration might stimulate growth of the pleuropneumonia-like microorganisms and thus increase the severity of the arthritis. Fildes and Richardson[9,10] have shown that *Staphylococcus aureus* is able to utilize some mercapto compounds as a source of sulphur and, in fact, that sulfhydryl compounds are essential metabolites for many cells. Thus, if lack of sulfhydryl groups prevents the metabolism of bacterial cells it is possible that an abundance of these *SH* groups as furnished by BAL might actually stimulate the microorganisms *in vivo*.

Conclusions. BAL(2:3-dimercaptopropanol), administered to rats with polyarthritis produced by infection with L-4 strain of pleuropneumonia-like microorganism, causes a significant increase in incidence of arthritis, a more extensive type of joint involvement, and a slightly decreased rate of survival. Concentrations of BAL up to 0.2 mg per cc of broth failed to inhibit or to increase the growth of pleuropneumonia-like organisms *in vitro*. It is suggested that BAL in the low concentrations used *in vivo* may act by furnishing sulfhydryl groups needed in the metabolism of the microorganisms, and thus aggravate the polyarthritis, rather than by poisoning the animals and thus reducing their resistance to the infection.

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[6] Peters, R. A., Stocken, L. A., and Thompson, R. H. S., *Nature*, 1945, **156**, 616.

[7] Waters, L. L., and Stock, C., *Science*, 1945, **102**, 601.

[8] Tripi, H. B., Kuzell, W. C., and Gardner, G. M., *Ann. Rheum. Dis.*, in press, June 1949.

[9] Fildes, P., and Richardson, G. M., *Brit. J. Exp. Path.*, 1937, **18**, 292.

[10] Fildes, P., *Brit. J. Exp. Path.*, 1940, **21**, 67.

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17107 P. Pyrogenicity of Influenza Virus in Rabbits.*

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During an investigation of the toxicity of influenza virus in rabbits, it was noted that the intravenous injection of infected chorio-allantoic fluid was followed by fever. To study this phenomenon, animals were placed in individual stalls, 3 preliminary rectal temperatures were taken at 30-minute intervals to establish a baseline, and the virus preparation to be tested was then injected into the marginal ear-vein. Rectal temperatures were recorded every 30 minutes throughout an observation period of 6 hours after injection. All glassware and saline solutions used were uncontaminated with bacterial pyrogens. A single pool of chorio-allantoic fluid from embryos infected with the PR-8 strain, having a hemagglutinin titer of 1:1024,[1] was used.

One ml of this material consistently caused a rise in temperature beginning 1½ to 2 hours after injection, reaching a peak of 3-4°F above the baseline in the next 4 hours, and gradually falling to normal. Although doses as small as 0.025 ml produced fever, the increases in temperature were of a lower order.

Normal chorio-allantoic fluid and infected fluid from which the virus particles had been removed by centrifugation at 30,000 R.P.M. or by adsorption on chicken erythrocytes gave no fever. The virus resuspended in normal saline solution produced typical temperature elevations.

No fever followed the injection of PR-8 virus neutralized with homologous immune serum.

The febrile response in rabbits is independent of the infectivity of the virus but seems to be related to its adsorptive capacity. Heating at 56°C for 30 minutes destroyed the infectivity of PR-8 for chick embryos but a portion of the hemagglutinin was retained (1024 to 128); this material produced definite fever in rabbits. Heating at 62°C for 30 minutes destroyed the hemagglutinin titer and these preparations were then non-pyrogenic.

The injection of the Lee strain of influenza B virus produced a fever in rabbits similar to that caused by PR-8. The hemagglutinin of Lee is known to be more heat-resistant than that of PR-8.[2] When the Lee strain was heated at 62°C for 30 minutes it retained adsorptive (1024 to 256) and pyrogenic properties. Exposure to temperatures which destroyed the hemagglutinin rendered the Lee strain non-pyrogenic.

It has been shown that rabbits given a course of daily injections of a bacterial pyrogen become tolerant to the fever-producing effect of that pyrogen and those of other bacterial strains.[3] Animals made tolerant by eight daily injections of 0.1 ml typhoid vaccine responded with typical fevers when given PR-8 on the ninth day, demonstrating that no cross-tolerance existed. The heat stability of bacterial pyrogens, which are not destroyed by boiling,[4] is also in contrast to the lower temperatures at which the fever-producing principle of influenza is destroyed.

Animals which had received 1.0 ml of PR-8 virus on the first day with attendant febrile responses were completely tolerant to the effect of an equal dose on the second day, when no temperature rise occurred. Nine animals given 1.0 ml of PR-8 were re-injected in groups of three on the fourth, seventh, and eleventh days and their temperature responses recorded. As seen in Fig. 1, the responsiveness to the fever-producing effect of the virus gradually returned so that by the eleventh day,

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* The opinions expressed are those of the authors alone and do not necessarily reflect those of the Navy Department or the naval service at large.

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[2] Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 134.

[3] Beeson, P. B., *J. Exp. Med.*, 1947, **86**, 29.

[4] Banks, H. M., *Am. J. Clin. Path.*, 1934, **4**, 260.

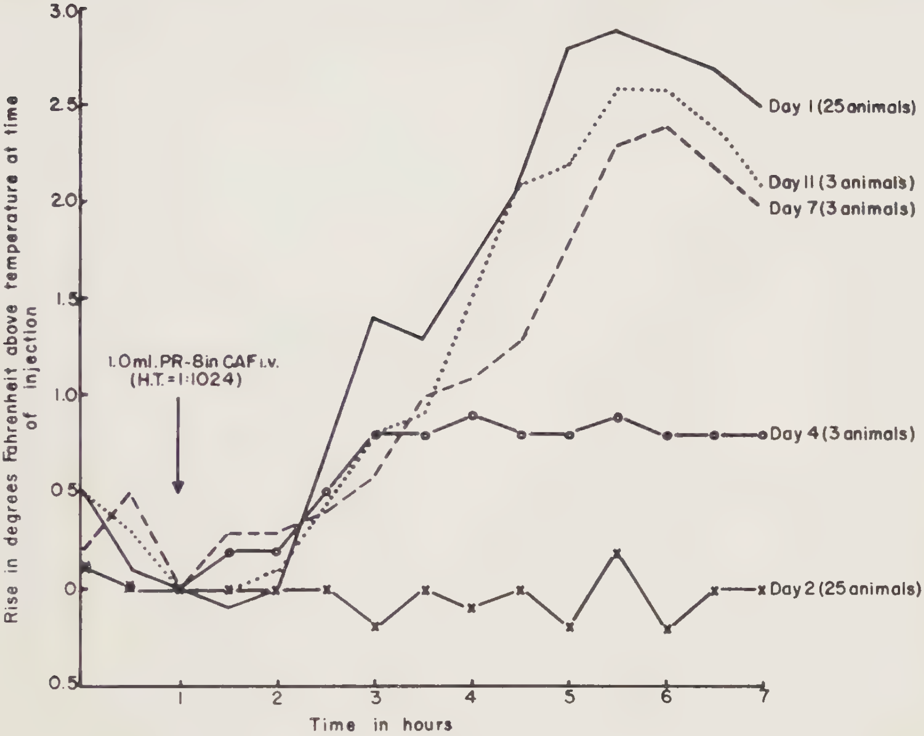

FIG. 1.

Average temperature responses of groups of rabbits to PR-8 influenza virus on day 1 and to a single injection repeated at intervals after the initial injection. Note the complete unresponsiveness on day 2 and the increase in fever through day 11.

their fevers approached first-day levels. Antibody titers (as measured by hemagglutination inhibition) of these animals showed increasing levels during this period when tolerance was being lost. Thus, there is evidence that the resistance to the pyrogenic effect of the virus is unrelated to specific antibody production, although as pointed out above, virus neutralized with immune serum before injection causes no fever.

The observation of Harris and Henle[5] that an injection of influenza virus will cause lymphopenia in rabbits was confirmed, but this effect on the lymphocytes was not seen on the second day. By the eleventh day, the lymphopenia, like the febrile response, again approached that following the first injection.

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17108. Biologic Absorption of Insolubilized Gelatin Films.

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Our experience in preparing absorbable hemostatic sponges from gelatin[1,2] encouraged us to investigate the possibility of developing a gelatin film that might also be clinically useful. During the course of our studies Bing[3] reported his observations using gelatin sponge at the end of which paper he mentionel without elaboration an incomplete study involving a gelatin film preparation.

The purpose of this report is to submit the data we have obtained from studying the *in vitro* water solubility and enzymatic digestion and *in vivo* biologic absorption of uniquely prepared gelatin films.

Several materials, both absorbable and nonabsorbable, have been employed in surgery as films. Brown, *et al.*[4] in their report on the use of polythene film mentioned most of these and noted the limitations of each. Oppenheimer's[5] report suggests that nonabsorbable substances must be observed for a sufficient period to assure that they are not carcinogenic since these investigators noted that cellophane implants induced sarcomas in rats after remaining *in situ* more than 11 months.

Materials and Methods. Films were prepared from commercial pigskin gelatin. To remove cations each 100 g of raw, ground gelatin was eluted for 2 hours with one liter of cold (5°C) M/128 glacial acetic acid; the acid was drawn off and the gelatin mass washed several times with cold double distilled water.

Usually 5% solutions were prepared from this washed gelatin. To these were either added nothing or various small quantities of U.S.P. formalin. The mixture was incubated at 37°C for 2 hours after which films were cast, dried on stainless steel plates under controlled humidity and heated at 140°C for different lengths of time. The investigations which are reported here were carried out on films that were approximately .003 inch thick. Films of such light weight, even when dry, are remarkably pliable and only break when folded sharply.

To obtain sterile preparations, when desired, the gelatin films were subjected to routine dry heat (140°C for 4 hours) sterilization in accordance with the official U.S.P. method.[6]

Three criteria were employed to establish to what degree a given treatment had altered the gelatin molecule yielding films with varying physical and biologic characteristics.

The first of these was the tendency of the film to go into water solution. To uniform quantities of film was added an appropriate volume of distilled water which stood on the gelatin at room temperature overnight. The next morning the solution was heated on a steam bath with occasional stirring and the films observed until dissolved or for at least 24 hours.

In another test the time for *in vitro* digestion[1] of films by pepsin was determined. To pieces weighing 150 mg was added 100 cc of a 1% U.S.P. pepsin solution. The containers were placed in a water bath held at 37°C and mechanically agitated; the time was noted when the last fragment had been digested.

Finally biologic absorption and possible reaction were investigated by implanting pieces that weighed roughly 15 mg (ca. 10 x 12 mm) usually in muscle layers, in the legs of rats using a technique similar to one that has been previously[2] described. Sixteen albino rats weighing around 300 g were prepared in each series.

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[6] Pharmacopeia of the United States, thirteenth revision, Mack Publishing Co., Easton, 1947.

TABLE I.
In vitro and *in vivo* Evaluations of Absorbable Gelatin Films.

Film preparation		Response in hot water	Proteolytic digestion time	Biologic absorption
No.	Treatment			
B-98-1	Untreated	In solution in 1 to 2 min.	Film soluble	Gone in less than 24 hrs.
B-98-2	Heated 2 hrs	In solution in 7 to 8 hrs.	70 to 80 min.	
B-98-3	Heated 4 hrs	Disintegrated into fragments in 24 hrs.	1½ to 2 hrs.	Gelatinous in 1 day; gone in 4 days
B-93-1	.03% formalin; not heated	In solution in 30 min.	40 min.	
B-93-2	.03% formalin; heated 4 hrs	Much swelling; no solution in 24 hrs.	2 to 4 hrs.	
B-93-3	.03% formalin; heated 8 hrs	Some swelling; no solution in 24 hrs.	5 to 8 hrs.	Gelatinous in 2 weeks; gone in 7 to 8 weeks
B-97-1	.06% formalin; not heated	In solution in 25 min.	60 to 80 min.	
B-97-2	.06% formalin; heated 4 hrs	Some swelling; no solution in 24 hrs.	5 to 8 hrs.	Gelatinous in 2 weeks; gone in 7 to 8 weeks

Results. The data obtained from several different investigations have been organized into a single table (Table I) for presentation. Values are reported in ranges to indicate that the tests cannot be reproduced absolutely from one experiment to the next, but that they do indicate direction. Thus it can be predicted that a film which will finally go into solution in water in 7 to 8 hours (B-98-2) will be digested by pepsin more rapidly than a film which only swells and will not dissolve in water (B-93-2).

It is our experience also that insolubilized films with short enzymatic digestion times (B-98-1, B-98-3) will soften and be resorbed in the body at a faster rate than those with appreciably longer (B-93-3, B-97-2) *in vitro* times.

Enzymatic digestion times and biologic absorption do not, however, have a straight line relationship. For we have observed with gelatin films, as Morrison and Singer have previously noted with fibrin films,[7] that variations in the mode of preparation may result in greatly prolonged *in vitro* digestion times with inconsiderable if any variation in the observed *in vivo* resorption. In experiments not recorded in the table we have prepared insolubilized gelatin preparations which remained undigested by pepsin after 24 hours yet were absorbed biologically in periods of time only slightly longer than films which were insolubilized to a lesser degree according to their *in vitro* characteristics.

Resistance to water solubility was imparted to the film by hot air heating of the dried film (B-98-2, B-98-3) or by the addition of minute quantities of formalin (B-93-1, B-97-1) to the original solution. A combination of the two techniques resulted in a greater degree of insolubilizing (B-93-2, B-93-3, B-97-2). From the table it can be seen that, generally, the more intensive the treatment by either or both of the methods the more altered the film according to the tests that were applied.

Biologic implants of films that had *in vitro* proteolytic digestion times of less than 4 hours softened in the muscle tissue rapidly and no residue could be detected upon gross inspection by the fourth day (B-98-3).

Because it seemed likely that such films would be clinically impractical, preparations were made that were more resistant to *in vitro* digestion. *In vivo* these were found to withstand undue disintegration for 8 to 10 days, to be gelatinous in about 2 weeks and be absorbed in 7 to 8 weeks (B-93-3, B-97-2).

In the series in which gelatin film B-98-3 was implanted in the left thigh muscles a comparable piece of regenerated cellulose (Du Pont cellophane No. 600 PT) was implanted in the same area in the right side. On inspection as long as 11 weeks after implantation the cellophane was readily located encapsulated in a thin membranous envelope; it showed no evidence of disintegration or resorption. Lowry[8] reported a tendency of certain cellophane films which he studied to be absorbed. In the studies of Oppenheimer *et al.*[5] the regenerated cellulose which they implanted was unabsorbed over a period of 11 months or more.[9]

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[7] Morrison, P. R., and Singer, M., *J. Clin. Invest.*, 1947, **26**, 929.

In addition to the gross observations which are recorded here, specimens of the implant areas were fixed and submitted for histologic examination; the results of the histologic readings will be reported in detail in another paper. Since the properties of water insoluble gelatin film suggested that it might have clinical utility wherever an absorbable membrane is indicated, this possibility is being independently investigated in several surgical research laboratories.

Summary. The preparation and properties of gelatin films, which resisted water solution in varying degrees, have been described. These films, although water insoluble, have been shown to be digested in different lengths of time when subjected to proteolytic enzymes *in vitro* in a standardized assay.

Some of the altered gelatin films were implanted into muscle areas of albino rats. Upon visual inspection they were found to be absorbed at the recorded intervals, with no evidence of untoward tissue reaction. The data indicate that the biologic absorption time can be roughly predicted from *in vitro* digestion results; as the *in vitro* times become greatly prolonged, however, a comparable resistance to biologic absorption is not seen.

Cellophane implants were not absorbed but at the eleventh week were encapsulated in a thin membranous capsule.

The authors are grateful to Margaret Borst for her assistance in this work.

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[8] Lowry, M. L., *Arch. Surg.*, 1946, **52**, 160.

[9] Oppenheimer, B. S., personal communication, November 1948.

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17109. Sodium Acetate as a Source of Fixed Base.*

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Recent studies have shown that acetic acid is an extremely rapidly oxidized product of the intermediary metabolism of both carbohydrate and fat.[1] From the large turnover of acetate in the normal metabolic processes of the body, it is obvious that sodium acetate should serve as a rapidly available source of fixed base. Using the change of serum CO_2 content as an index of the presumptive rate of oxidation of the acetate ion, studies were performed in dogs and in man to define the usefulness of sodium acetate as a source of fixed base.

Procedure and results. Dogs, anesthetized with pentobarbital, were infused intravenously with a solution of 1/6 molar sodium acetate, the total amount administered being 15 mEq per kg of body weight. Blood specimens were taken under oil at intervals of 15 minutes or longer, and serum CO_2 was determined by the manometric method of van Slyke. Studies on 8 dogs yielded uniform results and showed a rapid rise in CO_2 content. The observations from 5 experiments with similar rates of infusion are given in Table I. Within 15 minutes after starting the sodium acetate infusion the Serum CO_2 showed a prompt elevation and continued to rise during the infusion, leveling off in the afterperiod without further significant change. Infusion of the same

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* Aided in part by a grant from the United States Public Health Service.

† National Research Council Fellow.

[1] Bloch, K., *Physiol. Rev.*, 1947, **27**, 574.

TABLE I.
Effect of Sodium Acetate Infusion on Serum CO_2 Content in the Dog.

Dog	A	B	C	D	E	Avg
Duration of infusion (min.)	75	75	55	65	60	66
Time after starting infusion (min.)	Serum CO_2 volumes %					
0	51.5	51.0	53.7	56.2	52.4	53.0
15	60.4	62.4	64.8	68.7	64.1	64.1
30	74.1	75.0	82.0	80.2	75.6	79.4
60	88.8	89.3	92.6	99.8	99.2	93.9
120	97.0	97.1	100.2	105.8	102.2	100.5
180	97.0	98.1	104.1	107.4	102.1	101.7
240	—	98.8	104.0	106.8	102.0	102.9

quantity of sodium acetate twice as quickly promoted a rise which was less marked, thus suggesting that the renal threshold for acetate had been exceeded. Because of the reported vasodilator action of acetate,[2] blood pressures and electrocardiograms were taken, but no changes or abnormalities were noted with the doses employed.

Solutions of 1/6 molar sodium acetate were prepared and autoclaved in the routine manner for use in patients. Normal adult subjects and patients with renal acidosis were studied in the fasting state. Infusions of 1 liter were given within a one hour period. The average dosage, uncorrected for differences in weight of patients, was 2.8 mEq per kg of body weight, approximately one-fifth of the amount given per kg to the dogs. No attempt was made to define the upper limit of tolerance in human subjects. The amounts used satisfy reasonable clinical requirements. Blood pressures and pulse rates were determined at frequent intervals and no significant changes were noted, nor were any subjective symptoms or other evidence of toxicity observed. The results of the serum analyses are shown in Fig. 1. The rapid rise of serum CO_2 was evident within 15 minutes and the rise continued progressively. Except in 2 subjects, there was no further increase in serum CO_2 after termination of the infusion. The data have been presented without correction for body weight, body water or renal excretion, and although small variations could be accounted for by these factors, it is evident that acetate is rapidly metabolized with the release of fixed base. Three subjects were given one liter of 1/6 molar sodium bicarbonate in one hour as a control study. As seen in Fig. 1, the rate of increase in serum CO_2 content was essentially the same for the sodium bicarbonate infusion as it was for the sodium acetate, indicating, within the limits of the methodology employed, that there was an almost instantaneous utilization of the acetate. In 3 patients the urine became sharply alkaline fol-

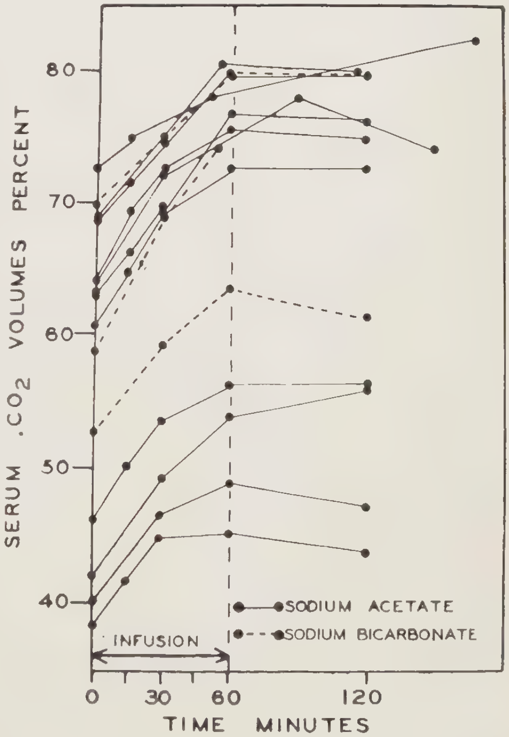

FIG. 1.

Comparison of effect of sodium acetate and sodium bicarbonate infusions on serum CO_2 content in man.

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[2] Bauer, W., and Richards, D. W., Jr., *J. Physiol.*, 1928, **66**, 371.

lowing the infusion of the sodium acetate.

Discussion. Sodium acetate affords a readily procurable and inexpensive source of fixed base, as rapidly and as completely available as sodium bicarbonate. Solutions are easily prepared, easily sterilized, and indefinitely stable. The pH of 5 different lots of sodium acetate made up in 1/6 molar concentration was found to vary from 7.6 to 8.0. We believe that the use of sodium acetate solutions is indicated in rapid parenteral alkalinization and should be useful in the therapy of most types of metabolic acidosis. However, we wish to emphasize that because the metabolism of acetate has not been specifically defined as to its possible effect on ketone body production in diabetic acidosis, we have not used it to date in this condition.

These studies have not revealed previously undescribed properties of sodium acetate. Indeed the salt has been recommended in standard textbooks[3,4] as a source of fixed base, but its clinical use has been limited. The present observations demonstrate the rapid catabolism of acetate and emphasize certain practical advantages which it possesses for clinical use.

Summary. Studies in dogs and in man demonstrate that sodium acetate is an easily prepared and a rapidly available non-toxic source of fixed base suitable for parenteral administration when alkalinization is indicated.

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[3] Goodman, L., and Gilman, A., The Pharmacological Basis of Therapeutics, The Macmillan Company, New York, 1941.

[4] Sollmann, T., A Manual of Pharmacology, W. B. Saunders Company, Philadelphia, 1948.

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17110. A Method for Assay of Serum Proteolytic Activity.*

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That blood contains an inactive precursor (plasminogen) which can be activated to form a proteolytic enzyme (plasmin) has been established.[1-7] Study of variations in this enzyme have largely been on the basis of its fibrinolytic properties. Using a method of preparation outlined by Milstone,[6] we have been able to obtain from serum a plasminogen-containing fraction with consistent and reproducible activation in humans and dogs.

Reagents and Materials. 1. *Streptokinase*—the filtrate of a 24-hour culture of Group A, Type 11, Beta hemolytic streptococci, grown in beef heart infusion broth with 0.05% added dextrose.

2. *Protamine Sulphate*§—a solution of protamine sulphate in veronal buffer, 2 mg per ml.

3. *Veronal Buffer*—pH 7.35[8]

4. *Acetic acid—distilled water mixture*—19 volumes of distilled water plus 0.32 volumes of 1% acetic acid.

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* Aided by a grant from the Reckford Research Fund of the Yale University School of Medicine.

† Fellow of Department of Surgery (Oncology), Yale University School of Medicine.

‡ Senior Fellow, American Cancer Society, as recommended by the Committee on Growth of the National Research Council.

[1] Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.

[2] Christensen, L. R., *J. Gen. Physiol.*, 1946, **30**, 149.

[3] Ratnoff, O. D., *J. Exp. Med.*, 1948, **88**, 401.

[4] Delezenne, C., and Pozerski, E., *Compt. rend. Soc. Biol.*, 1903, **55**, 690.

[5] Kaplan, M. H., Tagnon, J. H., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 533.

[6] Milstone, H. J., *J. Immunol.*, 1941, **42**, 109.

[7] Ferguson, J. H., Travis, B. L., and Gerheim, O. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 285.

§ Eli Lilly and Company, Indianapolis, Ind.

[8] Clark, D. G. C., Cliffton, E. E., and Newton, B. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 276.

TABLE I.
Sample Reading of Results in %T and Tyrosine Produced After 2, 6, and 24 Hours Incubation with Streptokinase (S), Protamine (P), and Spontaneously (O) Activated Plasminogen. Controlled by Reagent Blanks with 0.5 ml Veronal Buffer Substituted for Plasminogen.

Patient	Activator	% T			Tyrosine—mg		
		2 hr	6 hr	24 hr	2 hr	6 hr	24 hr
B.U.	S	79.5	45	6	.148	.256	.905
	P	91.8	100	59.6	.026	0	.166
	O	93	93.8	84.7	.023	.019	.053
H.D.	S	85	47	7.5	.050	.242	.835
	P	100	90	72	0	.033	.105
	O	96.4	88.5	81	.01	.039	.066
J.S.	S	90.8	86.5	61.5	.03	.045	.155
	P	94	90	58	.018	.033	.175
	O	95	93.5	65	.015	.02	.138
F.G.	S	58.2	24	4.5	.174	.458	1.0
	P	82	69	46.5	.063	.118	.245
	O	80.2	74	58.5	.07	.095	.173
H.C.	S	89	59	18.3	.036	.17	.545
	P	91.2	87.5	69.5	.028	.043	.116
	O	91.3	89.5	86	.027	.035	.048
Reagent blanks	S			100			0
	P			97			.008
	O			100			0

5. *Trichloracetic Acid* (USP), 16%

6. *NaOH* (Reagent grade), 1N

7. *Casein*—a 5% solution, adjusted to a final pH of 7.35-7.45.

8. *Folin-Ciocolteau phenol reagent*—diluted 1:3 with distilled water.

9. *Serum*—obtained from nonhemolyzed blood drawn under sterile conditions.

Method. Plasminogen is prepared by adding 1.5 ml serum to 28.5 ml acetic acid-distilled water mixture, inverting once to mix, and centrifuging at 2000 RPM for 15 minutes. The supernatant is discarded and the precipitate dissolved in 1.5 ml veronal buffer; 0.5 ml of this solution, containing plasminogen, is then added to each of three test tubes. To the first, 0.4 ml streptokinase and 0.1 ml buffer are added; to the second 0.5 ml protamine sulphate solution; and to the third, 0.5 ml buffer. 5 ml of casein solution are added to each tube, mixed by inversion, and a 2.5 ml aliquot taken immediately.

5 ml trichloracetic acid are added to each aliquot, and the resulting coagulum is separated from the sides of the tubes by thorough shaking. Each sample is filtered through Whatman No. 3 filter paper, and a 2.5 ml aliquot of the filtrate is added to 5 ml of 1N NaOH in a 50 ml Erlenmeyer flask. 1.5 ml of diluted phenol reagent is added to each flask in turn as rapidly as possible, and the contents of the flasks are transferred to spectrophotometric cuvettes. Exactly ten minutes after phenol reagent is added to the first flask, the %

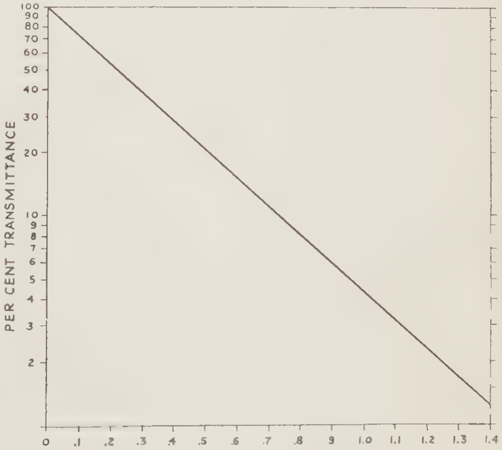

FIG. 1

Calibration curve. Standard tyrosine solution in casein.

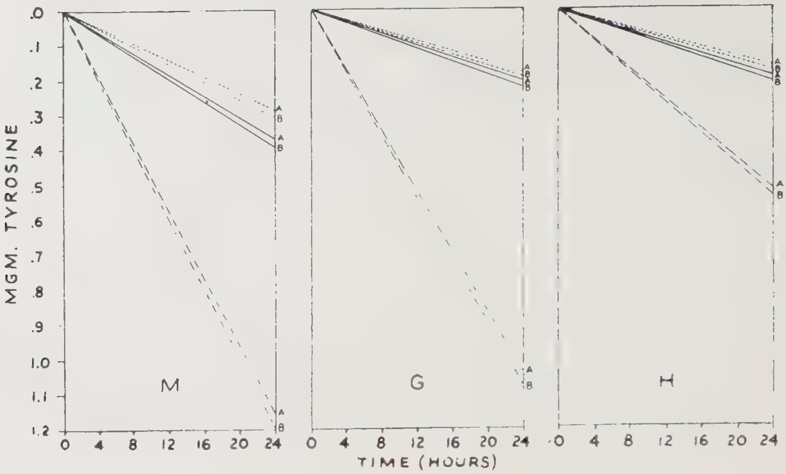

FIG. 2

Graphs of results with duplicate samples of blood of 3 pts. M, G, and H A and B represent aliquots of each serum taken simultaneously and treated as individual sera.

- - - - - - - - Spontaneous activity.
———————— Protamine activated.
— — — — — — Streptokinase activated.

transmittance of each sample is determined in turn in the Coleman Spectrophotometer, set at 675 mu. The original tubes are incubated for 24 hours at 37°C.

The second 2.5 ml aliquot is taken after 24 hours incubation at 37°C, and color is developed as described above. When the samples are read, the first aliquot acts as the "blank" for the second. A stock solution of a blue dye, T-1824, is prepared so that its optical density is greater than the optical densities of the first aliquots of the unknowns. In reading the first aliquots in the spectrophotometer, the color-producing agents already present in the digest mixtures are negated by setting the galvanometer index at 100% T, or 0 concentration. With this as a reference point, the %T of the dye is then taken for each sample. When the corresponding 24-hour sample is read, the dye, as the "blank", is set at the T value obtained the day before, and the % T of the unknown is then read directly.

Calculation. Standard amounts of tyrosine were added to 5 ml of casein solution, and 2.5 ml aliquots were taken and treated as above. These were read against a similar aliquot of casein without added tyrosine. A calibration curve was prepared, so the % T of unknown samples can be expressed directly as mg of tyrosine produced (Fig. 1, Table 1).

Results and Discussion. Aliquots of sera tested after 2, 6 and 24 hours' incubation showed steadily increasing amounts of tyrosine produced, and for experimental purposes the 24 hour period was chosen (Table I). Reproducibility was established by taking aliquots of serum simultaneously and treating them as individual samples (Fig. 2).

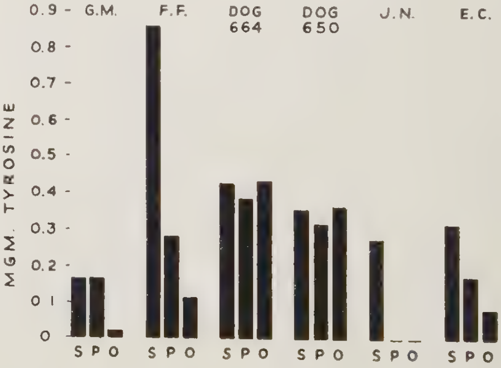

FIG.3

Columnar graphs showing activation by streptokinase (S), protamine (P), and spontaneous activation (O). 4 representative patients and 2 dogs. Note similarity of activation by agents in dogs as compared to humans.

Demonstration of the activation of plasminogen by streptokinase and the slow spontaneous activation confirms previous studies of this relationship. The activation by protamine is definite, though in most instances not as great as is that by streptokinase (Fig. 3). This is at variance with the previous impression that the fibrinolytic activity of protamine was due to inactivation of antifibrinolysin.[10] In the present study protamine sulphate (up to 1.2 mg/tube) failed to diminish antiproteolytic activity tested by the method previously reported.[8]

Dog sera differ from human in that their spontaneous activation is of the same order as the protamine and streptokinase activation (Fig. 3).

Summary. A method for preparation of plasminogen from small quantities of blood has been described. Activation by streptokinase, protamine sulphate, and spontaneous activity have been observed. Studies of other activators and of species differences are in progress.

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[10] Scroggie, A. E., Jaques, L. B., Rocha e Silva, M., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**. 326.

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17111. Automatic Measurement of Alveolar CO_2 in Small Animals.

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The following paper will describe the application of an infra-red analyzer to alveolar carbon dioxide studies in small animals such as the rabbit, where rapid respiration and small respiratory volumes pose severe problems. As reported elsewhere,[1] this analyzer has been used successfully in measuring the alveolar CO_2 tension in humans during hyperventilation as it is performed in routine electroencephalography. The analyzer was developed for other purposes by Luft[2] and applied to CO_2 measurement by Schmeiser.[3] It measures the infrared absorption of one gas in a mixture by using a fixed quantity of the same kind of gas as a thermomechanical metering device. This method allows an analysis which is rapid enough to fully record a change in CO_2 tension in one-seventh of a second. Thus, in human studies, alveolar air sampling devices are unnecessary, and a portion of the expired air is drawn continuously through the analyzer. The latency of measurement then depends solely upon the speed of transport of this air through the test chamber. The only additional equipment required is a long expiration tube to prevent dilution of the continuous sample by outside air. The measured CO_2 value after a certain expired volume can be safely regarded as the conventional "alveolar CO_2 tension."

Fig. I illustrates the CO_2 analyzer in simplified form. A hot-wire infra-red source (S) projects a beam of infra-red through a transparent (mica) test cell (T) containing the air for CO_2 analysis. The remaining infra-red is absorbed in a following chamber (A), one wall of which is a thin metal membrane. The temperature increase due to the absorption displaces the membrane by increasing the pressure, thus measuring the amount of infra-red reaching the chamber. If the chamber is filled with CO_2, it becomes sensitive to only those wave lengths which the CO_2 in the test cell absorbs. Thus, it is insensitive to any other gases appearing in the test cell. To balance the system, an identical arrangement (S′, C, A′) is attached to the other side of the membrane, and the two sides "buck" each other. Displacements of the membrane are measured by utilizing it as one plate of a condenser and

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[1] Blinn, K. A., and Noell, W. K., *EEG Clin. Neurophysiol.*, 1949, in press.

[2] Luft, K., *Z. f. Tech. Phys.*, 1943, **24**, 97.

[3] Schmeiser, K., unpublished work.

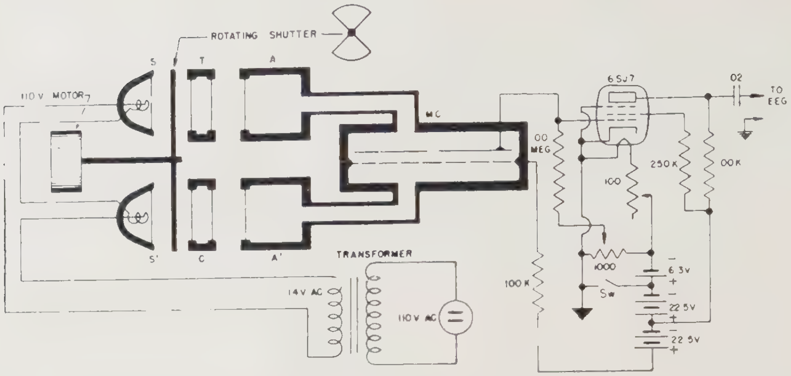

FIG. 1.
Diagram of the CO_2 analyzer and its associated preamplifier. S,S′—hot wire infra-red sources; T,C—test and control chambers; A,A′—absorption chambers of membrane-condenser, MC.

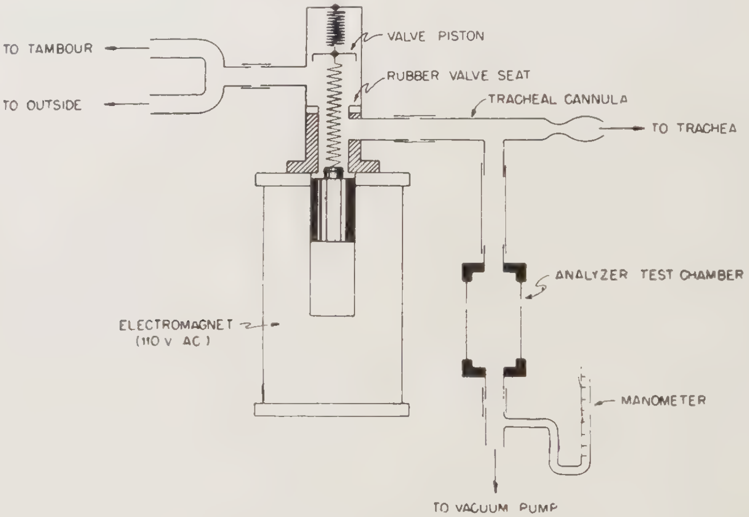

FIG. 2.
The alveolar gas sampling apparatus. The connections between the electromagnetic valve, the tracheal cannula, and the analyzer test chamber are made as short as possible.

measuring changes in electrical charge on the condenser. To produce an alternating voltage for amplification, the two infra-red beams are simultaneously interrupted 7 times per second by a rotating shutter. After amplification the output of the analyzer can be recorded on any instrument, for example, an electroencephalograph. The output appears as a train of 7c/s waves, the amplitude of which is proportional to the CO_2 concentration within limits determined by the thickness of the test cell.

Respiration in humans, even during hyperventilation, is conveniently slow; but in small animals, such as the rabbit, it may be so rapid that the seven-per-second shutter frequency is proportionally too low, and the respiratory volume exchange becomes a critical factor. Unfortunately, the shutter frequency can be increased only at the expense of a major part of the sensitivity. In these animals, the respiratory rate may exceed 200/minute. Therefore, in order to obtain an undiluted alveolar sample in the test chamber, it was necessary to artificially lengthen a single expiration whenever such a rate prevailed. Fig. 2 shows how this was managed. A tracheal cannula

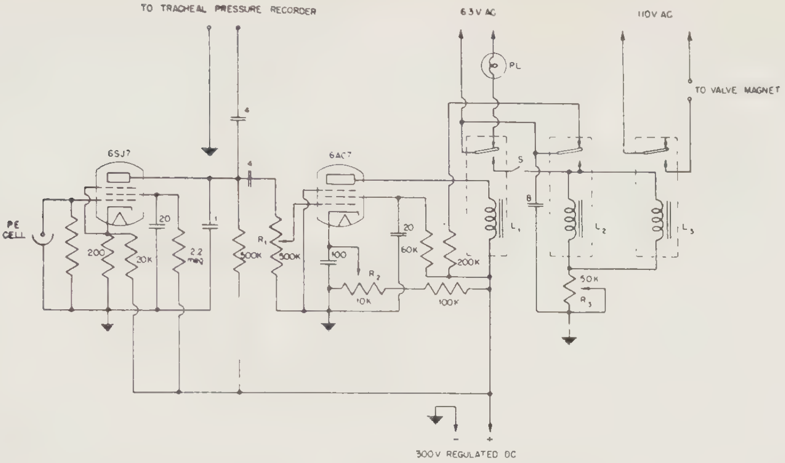

FIG. 3.

The electronic circuit for activating the valve. Bias is adjusted beyond cut-off with R_2; R_1 is then advanced until the pilot light PL begins to extinguish at the peak of expiration. When the switch S is closed, the valve magnet is activated for a single sampling of predetermined duration. R_3 varies the sampling duration from ⅓ sec. to 2 sec. L_1, L_2, and L_3 are 10,000 ohm relays.

was used, with a side arm leading to the test chamber and suction pump. To obviate disturbances of the respiratory rhythm and vagus effects, an automatic system was devised for briefly closing the cannula at the peak of the expiratory pressure curve. An electromagnetic valve closes the distal end of the tracheal cannula so that an alveolar sample is drawn through the analyzing chamber by the pump. The onset and duration of the closing are controlled electronically in the following manner: tracheal pressure fluctuations are transformed into voltage fluctuations by means of a tambour-operated shutter in front of a photoelectric cell. The electrical fluctuations operate a series of relays (Fig. 3) in such a manner that when a sampling switch is closed, the electromagnetic valve closes at the peak of the next expiration and remains closed only for a preset length of time.

Fig. 4 illustrates the result. The CO_2 is recorded on the electroencephalograph tape simultaneously with cortical potentials, the EKG, and the tracheal pressure. Each strip is about 12 seconds long. In the top example, the respiration rate is about 180/minute, and the short groups of waves represent the CO_2 concentrations over single expirations. The highest amplitude varies from one group to another because of the varying

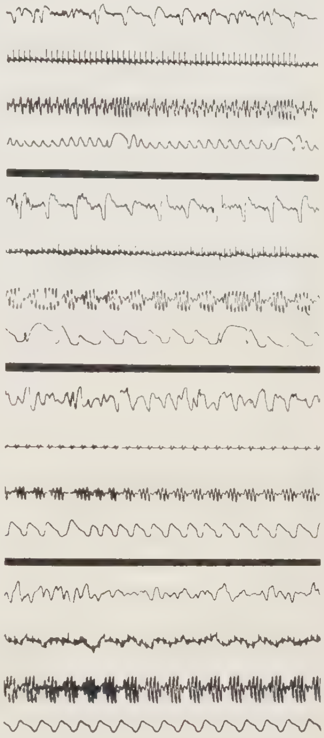

FIG. 4.

Ink-writer recordings illustrating alveolar CO_2 measurements in a rabbit. From top to bottom, the tracings in each strip are: electrocorticogram (area striata), EKG, CO_2 tension, and tracheal pressure. Description in the text. The cortical activity is being driven by one-per-second photic stimulation.

amounts of alveolar air caught in the test chamber. The 3 long groups are samples taken automatically. It can be seen that such sampling was necessary at this respiration rate. During the sampling, the heart rate and electrocorticogram do not change, indicating that there were no appreciable disturbances by vagus reflexes. The next example shows a slower respiration of about 60/minute, and here it is obvious that the sampling was unnecessary. The CO_2 concentration does not become any higher during prolongation of expiration. The third example demonstrates conditions under severe hypoxia, which was induced shortly after example 2. The alveolar CO_2 has dropped considerably, and it remains low throughout the gasping period which follows until oxygen is resupplied. The immediate increase to a very high CO_2 concentration is shown in the last strip, which was recorded during post-anoxic convulsions. Under the conditions of the last two examples, automatic sampling was proven unnecessary and even undesirable because of increased vagus excitability. When such a high excitability is combined with a high respiration rate, the described sampling method is not feasible.

Summary. An infra-red CO_2 analyzer having a measurement rate of seven analyses per second is described for continuous CO_2 tension determination over the expiration time (or volume). The value recorded from the last portion of expired air is regarded as "the alveolar CO_2 tension" if a plateau has developed during expiration. Respiration in small animals is often so rapid (relative to the measurement rate) that forced prolongation of a single expiration is necessary in order to draw undiluted alveolar air into the analyzer. A simple method is described for accomplishing this by means of a valve whose closing is electronically initiated and timed.

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17112 P. Multiplication of the T3 Bacteriophage Against *E. coli.*

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Steps in the development of new bacteriophage particles from diseased bacteria are particularly well seen in electron micrographs of young and frequently transferred colon bacilli infected with the T2 strain of their bacteriophage.[1] Examination of such photographs has suggested that this bacteriophage is self-reproducing with a mode of multiplication that in some respects resembles that of coccoid bacteria. An unforeseen result of these observations has been the rapidity with which lysis, understood as rupture of the bacterial membrane, appears after infection with bacteriophage. It has usually been taken for granted that when a bacteriophage particle is adsorbed to a susceptible bacterium, it proliferates either at the surface or after penetrating the organism until, at the conclusion of the multiplication process, the cell bursts and frees the new particles that have been forming. Evidence from the electron micrographs demonstrates that the membranes of young colon bacilli in a growing culture rupture soon after mixture of the cells with enough T2 particles to disease them. On this basis the "burst interval" observed for these bacteriophages is determined by the dispersal of newly formed bacteriophage from the disappearing protoplasmic mass in which it has been developing.

Further light upon this and other aspects of the multiplication of bacteriophage has now been obtained by investigating the lysis of young *E. coli* by the two different bacteriophages, T1 and T3.

When a one-hour culture of the B strain of *E. coli* obtained by two or three transplants

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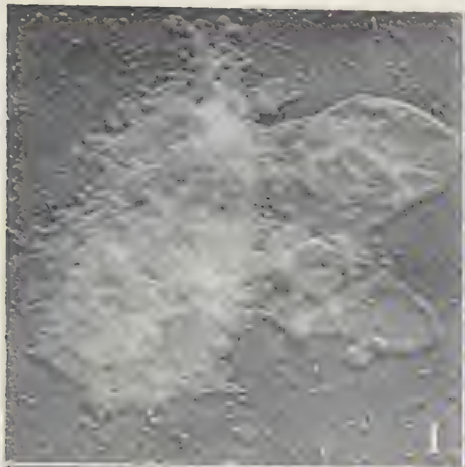
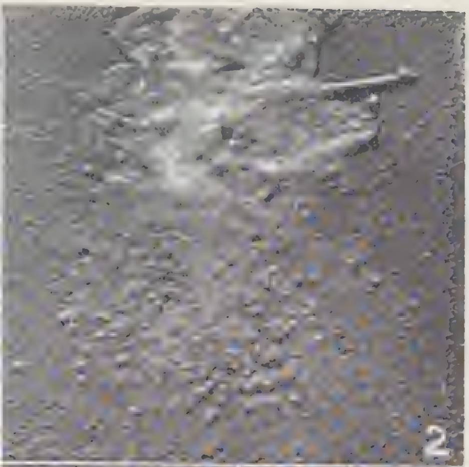
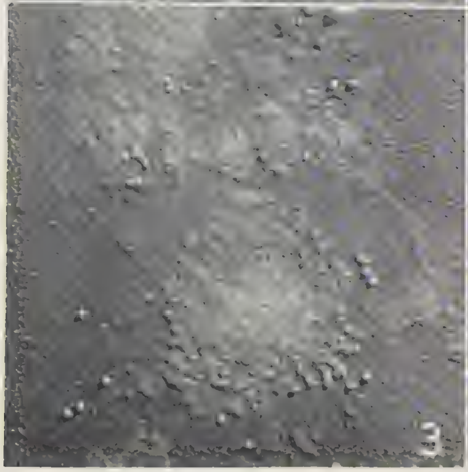
[1] Wyckoff, Ralph, W. G., *Nature*, 1948, **162**, 649.







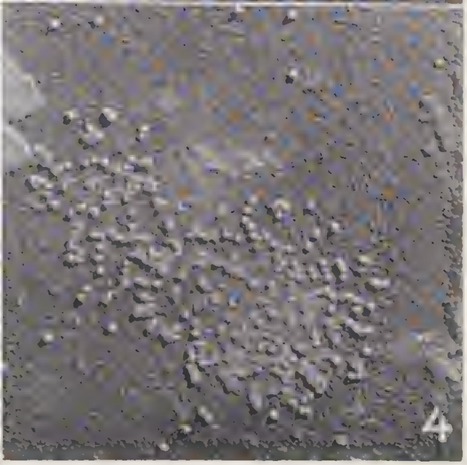


FIG. 1. A colon bacillus lysed through the action of T3 bacteriophage. From a culture after 5 minutes incubation. The cell membrane is at the right, the extruded granular protoplasmic contents at the left. Palladium shadowing on this and the following three preparations. Magnification, 16,667 ×.

FIG. 2. Particles of T3 phage developing within the protoplasm of a lysed colon bacillus. The empty cell membrane is at the top of the picture. Magnification, 16,667 ×.

FIG. 3. A somewhat more advanced stage in the development of a microcolony of T3 within protoplasm from a lysed bacterium. Magnification, 16,667 ×.

FIG. 4. A still later stage of phage production in which most of the cellular protoplasm has been consumed and the new particles are beginning to disperse. Magnification, 16,667 ×.

at hourly intervals has been mixed with enough T3 bacteriophage to infect at once all the bacteria present, electron micrographs have been obtained that agree with the earlier ones using T2 in showing prompt lysis; after 5 minutes incubation followed by immediate icing very few intact organisms have been found in centrifuged, formalinized and washed preparations. Such rupturing has not been observed in control preparations from which the bacteriophage has been omitted. When T1 bacteriophage of the same titre has been substituted for T3 many, but far from all the young cells in a culture have been quickly lysed. As Fig. 1 indicates, the bacterial protoplasm liberated by these bacteriophages is the same mass of spherical macromolecular particles seen after lysis with T2.

Preparations made after incubating for intervals up to half an hour following infection with T3 have shown various steps in the development of new bacteriophage particles in the protoplasm of infected and lysed cells. After 15 minutes incubation fields like those of Fig. 2, 3 and 4 are common. The lysed cell of Fig. 2 resembles that of Fig. 1 in showing its protoplasm extruded from the broken membrane. Much of this protoplasm has, however, already been converted into new particles of T3. The conversion is still more complete in Fig. 3 where the cluster of coccoid T3 apparently is developing from one or more central particles. This cluster recalls a bacterial microcolony resulting from several divisions of a single cell. A still later stage wherein the newly formed particles have commenced to disperse is shown in Fig. 4. After a few more minutes incubation the formation of bacteriophage has progressed so far at the expense of the lysed protoplasm that it is hard to find either the protoplasmic masses or the clumps of bacteriophage evident in these earlier photographs. Many electron micrographs like the foregoing have been obtained; they exhibit T3 particles in small groups and chains that suggest they have arisen by the same kind of self-reproduction which is often seen in bacterial growths and which appears to underlie the multiplication of the T2 coli bacteriophage.

Summary. Cultures of young, rapidly growing susceptible colon bacilli obtained after hourly transplants to fresh broth appear quickly lysed by the addition of T3 bacteriophage. Electron micrographs show that the bacterial membranes rupture very soon after adsorption of bacteriophage and that colonies of new phage particles rapidly appear in the protoplasmic masses. This protoplasm from young cells is the same assemblage of spherical macromolecules seen after lysis with T2 phage. As was the case with this other phage the observed phenomena are those to be expected of particles behaving as if they were minute microorganisms multiplying by division at the expense of the bacterial protoplasm.

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17113. Effect of Chemical Irritation of the Peritoneum on Transmural Migration of Intestinal Organisms.

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For reasons already given elsewhere[1] it is believed that the peritonitis which develops during peritoneal irrigation for the treatment of acute renal failure is due to the transmural migration of bacteria from the intestine. While there is no evidence that this can occur in the absence of severe damage to the intestinal wall, it might be possible as a result of the combined effect of the uremic state and the irritating properties of the dialyzing fluid. In the absence of clinical evidence for or against such a hypothesis, animal experiments were performed to see if transmural migration of intestinal bacteria can be induced (1) in normal animals subjected to chemical irritation of the peritoneum and (2) in normal and uremic animals exposed to the intraperitoneal injection of the fluids used for peritoneal irrigation in acute renal failure. The results of the first part of these studies are herewith reported.

Method. Healthy rabbits and dogs received sterile physiologic saline solution or the fluid used for peritoneal dialysis of uremic patients, in a volume sufficient to grossly distend the peritoneal cavity twice daily up to 2 weeks. Peritoneal fluid was aspirated as completely as possible just before each infusion. The as-

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[1] Frank, H., Seligman, A., Fine, J., *Annals Surg.*, 1948, **128**, 3.

TABLE I.

Effect of Intraperitoneal Injection of Sterile Monolate Solution and of Sterile Gum Tragacanth Suspension in Rabbits and Dogs.

Day	Monolate.																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Gross pathology
R_1 Injection (cc)	1	1	1	2	2		3	2	2	2	—	—	—	—	—						Fibrinous peritonitis.
Culture	—	—	—	—	+		+	+	+	+	+	+	+	—	—	S					
R_2 Injection (cc)	2	2	2	3	3	3		3	3	3											Fibrinopurulent peritonitis.
Culture	—	—	—	—	+	+		+	+	+	S										
R_3 Injection (cc)	1	2	2	2	2	2		3	3	3	3	3	3		3	3	3				” ”
Culture	—	—	—	—	+	+		+	+	+	+	+	+		+	—	+	S			
*R_4 Injection (cc)	2	2	2	2	2	2		2	2	2	2	2	2		2	2	3	4	4	—	Multiple adhesions. No active inflammation.
Culture	—	—	—	—	—	—		—	+	+	+	+	+		+	+	+	+	+	+	
*R_5 Injection (cc)	2	3	3	3	4	4		4	4	4	4	—	—		—	—	—	—	—	—	” ” ” ” ”
Culture	—	—	—	—	—	+		+	+	+	+	+	+		+	—	—	—	—	—	
*R_6 Injection (cc)	2	3	3	3	3	3		3	3	3	3	—	—		—	—	—	—	—	—	” ” ” ” ”
Culture	—	—	—	+	+	+		+	+	+	+	+	+		+	—	—	—	—	—	
*R_7 Injection (cc)	2	3	3	3	3	3		3	3	3	3	—	—		—	—	—	—	—	—	” ” ” ” ”
Culture	—	—	—	+	+	+		+	+	+	+	+	+		+	+	—	—	—	—	
D_1 Injection (cc)	4	5	5	5	5	8		8	—	5	5	5	5		—						Fibrinopurulent peritonitis.
Culture	—	—	—	—	+	+		+	+	+	+	+	+		+	S					
D_2 Injection (cc)	5	5	5	5	5	5		—													” ”
Culture	—	—	—	+	+	+		+	S												
D_3 Injection (cc)	5	5	5	5	5	5		5	—												” ”
Culture	—	—	—	+	—	—		+	+	S											
	Tragacanth.																				
R_1 Injection (cc)	10	10	—	—	—																” ”
Culture	—	—	+	+	+	S															
R_2 Injection (cc)	10	—	—	—																	” ”
Culture	—	—	+	+	S																
R_3 Injection (cc)	10	—	—																		Fibrinopurulent hemorrhagic peritonitis.
Culture	—	—	+	S																	
D_1 Injection (cc)	40	40	40	40	40	40		40	—	—	—	—	—		—	—					Subacute peritonitis.
Culture	—	—	—	—	—	—		—	—	—	—	—	—		—	—	S				
D_2 Injection (cc)	40	40	40	40	40	40		—	—												Fibrinopurulent peritonitis.
Culture	—	—	—	+	+	+		+	+	S											
D_3 Injection (cc)	40	40	40	40	—	—		—	—	—	—	—	—		—						Fibrinous peritonitis.
Culture	—	—	—	—	+	+		+	+	—	—	—	—		—	S					
D_4 Injection (cc)	40	40	40	40	40	40		40	—	—	—	—	—		—	—					” ”
Culture	—	—	—	—	—	—		—	—	—	—	—	—		—	—	S				

The volume of solution injected each day is indicated by the number. Blank spaces prior to S indicate that no injection or culture made on that day. R = Rabbit. D = Dog. — = Sterile culture. + = Presence of *E. coli in* peritoneal fluid. S = Sacrifice of animal.

* R_4—Cultures positive >6 days, followed by 8 negative cultures and sacrifice of rabbit on 36th day. R_5, R_6, R_7—Cultures negative >9 days and sacrifice of rabbits on 32nd day.

pirated fluid was always found devoid of bacteria by aerobic and anaerobic culture. A moderate leukocytic response was regularly present after the first few infusions. Postmortem examination of some of the animals after 2 weeks showed no grossly recognizable changes in the peritoneum.

A severe inflammation of the peritoneum was then produced in a second group of animals by the intraperitoneal injection of sterile 5% monolate (monoethanolamine oleate), which is strongly irritating to endothelial cells. Since a single dose of 4 cc of monolate killed rabbits within a few hours, a graded dose of from 1-4 cc was injected daily into each of a series of rabbits. Each of a series of dogs received 5 cc of monolate daily.

Another series of rabbits and dogs received a daily dose of 5 cc and 40 cc of sterile 2½% gum tragacanth suspension[2,3] respectively, for a number of days. Samples of peritoneal fluid were taken daily, with sterile precautions, from all animals. The animals were sacrificed on the day following the last injection or at some later date. When the tap was dry, 5 or 10 cc of sterile saline were injected and, after a few minutes, a portion of this fluid was withdrawn. All specimens were smeared and cultured immediately in tryptone broth, on agar and on endoplates. The same small loop was used for all inoculations, so that positive plates would permit a roughly quantitative measure of the degree of contamination. Broth and plates were observed for as long as 10 days.

Contamination of peritoneal fluid from the irritant itself or from the skin at the site of puncture was avoided by sterile technic and by the finding of no growth in cultures made from the irritant and from the skin. Daily blood cultures were also done on several animals throughout the experiment to exclude bacteremia as a source of peritoneal contamination. Identification of bacteria was made by standard bacteriologic technics.

Results Table I. Cultures of all animals injected with monolate or gum tragacanth if positive contained *Escherichia coli* and never any other organism. The number of colonies on direct plates was always less than 50 colonies per plate. *E. coli* was found regularly in the peritoneum of all rabbits and dogs beginning on the 4th to 9th day of injection. Once the microorganism appeared, it continued to be present until the injections were stopped. In four rabbits the culture remained positive for from 3 to 7 days after the last injection, and sterile for from 9 to 14 days thereafter. These rabbits showed extensive intraperitoneal adhesions without active inflammation, while those sacrificed shortly after the last injection showed diffuse fibrinopurulent and adhesive peritonitis. That the fibrinopurulent peritonitis was produced by the monolate rather than by *E. coli,* is indicated by finding in several rabbits and dogs sacrificed after only 2 or 3 days of monolate injections that cultures taken each day and at postmortem were sterile despite the presence of a marked fibrinopurulent peritonitis.

Gum-tragacanth Experiment (Table I): Three rabbits injected intraperitoneally with one dose of gum-tragacanth showed a fibrinopurulent peritonitis within 24 hours and *E. coli* was recovered at the same time. In dogs, however, the results were somewhat different. Four dogs, injected once, revealed sterile peritoneal cultures each day up to the time when they were killed on the 2nd, 4th, 5th, and 7th days respectively. At autopsy they all showed mild degrees of fibrinopurulent or fibrinous peritonitis. The results were the same in 2 more dogs which received daily doses of tragacanth for 4 days and for 7 days respectively. In another which received a daily dose of 40 cc of tragacanth for 6 days, *E. coli* first appeared on the 4th day and continued to be present in the peritoneal exudate for 4 more days. At postmortem he showed severe fibrinopurulent peritonitis. In still another dog similarly treated for 4 days *E. coli* was first found on the 5th day and continued to be present for 4 more days, after which sterile cultures were obtained during 5 subsequent days. Autopsy revealed a resolving fibrinous peritonitis. That the positive cultures were in no instance due to bacteremia, contamination from the skin at the

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[2] Steinberg, B., Goldbatt, H., *Int. Med.*, 1927, **39**, 449.

[3] Steinberg, B., *Am. J. Clin. Path.*, 1936, **6**, 253.

site of injection or of the irritant itself was shown by uniformly negative cultures from these sources.

Discussion. In spite of the large number of investigations which have been made on the permeability of the intestinal wall for bacteria, conclusive evidence of transmural migration of intestinal bacteria has not been produced. A normal intact serosa is generally regarded as impermeable, but this may not be the case where there is inflammation of the peritoneum due to a foreign body or a chemical irritant. For example, the presence of bile or a sponge in the peritoneal cavity may result in peritonitis or abscess formation, with organisms of intestinal origin present in the absence of a visible break in the continuity of the intestinal serosa. However, convincing proof that in such instances no break in continuity, however small, was responsible is difficult to produce.

Experimental data are contradictory. When a foreign substance, such as melted agar or sodium nucleinate[4] or broth[5] is placed in a serous cavity, intravenously injected cocci are found occasionally in the cavity containing the foreign substance but not in the other cavities. Thus an irritant in the peritoneal cavity may attract and retain intravenously injected cocci, but such organisms obviously must have entered the cavity via the circulation. Peritonitis following the feeding of virulent cocci usually does not occur. [6–12] In several instances where this did happen transmural migration was not proved, because bacteria may have entered the circulation via the inflamed intestinal wall.[13–17]

The recovery of the organism responsible for primary peritonitis from the intestinal wall[18,19,16] or in the stool[20,21] does not establish the case for transmural migration, because all of the involved areas may have been invaded from the blood stream. The evidence that the intestinal serosa when intact can be traversed by bacteria has therefore been equivocal.

In our experiments the sterility of the injected substance and of the skin, ascertained by daily cultures, exclude an external source for the bacteria found in the peritoneal cavity of our animals. Invasion via the blood stream is unlikely. To exclude this remote possibility daily blood cultures from two rabbits and one dog were taken during the entire time of the experiment and yielded no growth. In view of these observations the regular finding of a typical intestinal microorganism in pure culture in the peritoneum inflamed by chemical irritation is strongly in favor of its origin from the gut by transmural migration, in spite of the absence of any signs at postmortem examination of mucosal inflammation. Why only *E. coli* and none of the other intestinal bacteria was recovered is difficult to say. Pure *E. coli* peritonitis is rare.[22,23,13] The fact that *E. coli* was the usual contaminant or infecting organisms in patients subjected to peritoneal irrigation for uremia suggests that the mechanism of invasion in these patients was similar to that in the foregoing experiments.

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[4] Pawlowski, P., *Zentralblatt f. Chir.*, 1887, No. 48; *Virchows Arch.*, vol. 117, 1889, quoted by Künzel, H., *Münchn. Med. Woch.*, 1904, **51**, 1920-1921.

[5] Peiser, A., *Beitr. z. Klin. Chir.*, 1907, **55**, 484.

[6] Austerlitz, F., Landsteiner K., Sitzungberichte der kaiserlichen Akademie der Wissenschaften in Wien, 1889.

[7] Markus, H., *Z. f. Heilkunde,* 1899, **29**, 427.

[8] Neisser, M., *Z. f. Hyg.*, 1906, **54**, 363.

[9] Opitz, E., *Z. f. Hyg.*, 1904, **48**, 67.

[10] Selter, H., *Z. f. Hyg.*, 1896, **22**, 12.

[11] Bail, M., *Arch. f. Klin. Chir.*, 1900, **62**, 369.

[12] Hornemann, E., *Z. f. Hyg.*, 1911, **69**, 39.

[13] Lenander, K., Nyström, G., *Z. f. Klin. Med.*, 1906, **63**, 263.

[14] Geirsvald, S., quoted by Lenander, K. G., Nyström, G.[13]

[15] Felsen, J., Osofsky, A. G., Pharyngogenic Hematogenous Streptococcic Peritonitis, *Arch. Surg.*, 1935, **31**, 437.

[16] Weil, S. A., Saphir O., *Am. J. Dis. Child.*, 1932, **43**, 611.

[17] Montgomery, A. H., *Surg. Gyn. Obst.*, 1925, **21**, 798.

[18] Escherich, T., *Jahrb. f. Kinderheilkunde*, 1889, **49**, 167.

[19] Flexner, S., *The Johns Hopkins Hospital Bull.*, 1895, **6**, 64.

[20] Obadalek, W., *Deutsche Z. f. Chir.*, 1929, **220**, 307.

[21] Obadalek, W., *Deutsche Z. f. Chir.*, 1931, **233**, 587.

[22] Welsh, quoted by Flexner.[19]

[23] Fishbein, M., *Am. J. Med. Sciences*, 1912, **144**, 502.

Summary. Chemical peritonitis induced by repeated injections of monolate or gum-tragacanth in rabbits and dogs results in the appearance of *E. coli* in pure culture, in small to moderate numbers, in the inflamed peritoneum. Once this contamination is established, it continues for some time after the injections are discontinued. Eventually all cultures are sterile.

By exclusion of all other possible sources of contamination of the inflamed peritoneum, it appears that the *E. coli* found in the peritoneal exudate is derived from the intestinal lumen by transmural migration.

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17114. Transmural Migration of Intestinal Bacteria During Peritoneal Irrigation in Uremic Dogs.*

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Peritonitis due to *Escherichia coli* in pure culture occurs readily during peritoneal irrigation for the treatment of acute renal failure.[1] Clinical data suggested that the peritonitis was due to invasion of the peritoneum by the transmural migration of *E. coli* from the intestinal lumen.[1]

In a previous experimental study[2] it was demonstrated that transmural migration of *E. coli* can be induced by the intraperitoneal injection of strong irritants, but that bacterial peritonitis does not ensue. It was then considered desirable to determine whether the irrigating fluid used in treating acute renal failure can also provoke a bacterial invasion of the peritoneal cavity from the gut. The data to follow demonstrate that bacterial contamination is produced following intraperitoneal administration of this irrigating fluid in uremic dogs but not in normal dogs and that prophylactic oral antibiotic therapy is effective in preventing it.

Method. Normal dogs, and dogs made uremic by removal of both kidneys 24-72 hours previously, were treated as follows: Fifteen hundred to 3000 cc of sterile irrigating fluid‡ was injected into the peritoneal cavity by needle puncture under rigid sterile precautions. The fluid, as it escaped from the needle, just before and just after the puncture, was inoculated into dextrose broth. At the same time, swabs of the site of puncture of the skin, which had been prepared by shav-

	Solution A (g/l)	*Solution B* (g/l)
NaCl	7.25	7.4
KCl	0.19	0.05
$CaCl_2$	0.095	0.05
$MgCl_2 \cdot 6H_2O$	0.2025	0.22
$NaHCO_3$	1.5	0.77
Dextrose	9.5	5.0
Gelatin§	4.75	10.0
pH after sterilization	7.9	7.2-7.6

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* The work described in this paper was done under a contract, recommended by the Committee on Medical Research of the Office of Naval Research of the United States Navy and Harvard University.

† With the technical assistance of Sunya Gordon and Dorothy Kaufman.

[1] Frank, H. A., Seligman, A. M., and Fine, J., *Ann. Surg.*, 1948, **128**, 561.

[2] Schweinburg, F. B., and Heimberg, F., Proc. Soc. Exp. Biol. and Med., 1949, **71**, 146.

‡ The 2 solutions used for infusion were those which had been used in patients for peritoneal irrigation.[1] Each was used in about half of the experiments. As there was no difference in their effect, the results are not considered separately.

§ We are indebted to the Atlantic Gelatin Company for the gelatin used in these experiments.

TABLE I. Summary of Experiments.

Dog No.	Preliminary treatment		Infusion, days	Total vol. infused (cc) × 1000	Organisms in peritoneal fluid*		Cause of death	Gross peritonitis	
					E. coli, day	Others			
1	None		11	10.5	0	0	Distemper	No	
2	,,		13	16.9	0	0	Sacrificed	,,	
3	,,		16½	40	0	0	,,	,,	
4	Bilateral nephrectomy	(1)†	9	10	6‡	0	Uremia	,,	
5	,, ,,	(1)	4	9.4	0	0	Pulmonary edema	,,	
6	,, ,,	(1)	6	11.4	5	*A. aerogenes*	Uremia	,,	
7	,, ,,	(2)	9	19.2	7	,,	,,	,,	
8	,, ,,	(2)	11	30.1	9	,,	,,	,,	
9	,, ,,	(2)	14	44.9	7	,, Para-coli	,,	,,	
10	,, ,,	(3)	3	7.2	0	0	,,	,,	
11	,, ,,	(3)	5½	10.5	6	*A. aerogenes* *B. pyocyaneus*	,,	Fibrinopurulent peritonitis	
12	,, ,,	(3)	9	37.8	12	*A. aerogenes*	,,	No	
13	,, ,,	(3)	4	11.8	4	*A. aerogenes* *B. megatherium*	,, enteritis	Diffuse hemorrhagic peritonitis	
14	,, ,,	(3)	13	39.5	7	0	,,	No	
15	,, ,,	(1)	8½	39.2	8	0	,,	No; no foreign body reaction	Sterile rubber tubing in peritoneal cavity
16	,, ,,	(1)	2	5.5	0	0	Pneumonia	Fibrinous adhesions about foreign bodies	Sterile stainless steel and rubber tubing in peritoneal cavity
17	,, ,,	(2)	4½	14.5	3	*Strep. fecalis*	Uremia	No; no foreign body reaction	Same
18	Sulfathalidine and streptomycin orally 5 days, then bilateral nephrectomy	(2)	6	18.5	0	0	,,	No	
19	Sulfathalidine and streptomycin orally 4 days, then bilateral nephrectomy	(2)	5	17.8	0	0	,,	,,	
20	Same	(3)	12	44.7	0	0	Sacrificed	,,	
21	Same	(3)	9	38.6	0	0	Uremia	,,	

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* Organisms, when present, persisted from the day first found until death, with the exception of inconstant growth of *A. aerogenes* in Dog 9.

† Days before start of infusions.

‡ Days after start of infusions.

ing, alcohol and zephiran, were also inoculated into dextrose broth. To establish the initial sterility of the peritoneal cavity, fluid aspirated from the peritoneal cavity shortly after the first infusion was inoculated into dextrose broth, on agar and endo plates and into anaerobic media.|| Infusions were given twice daily and the foregoing procedures were repeated on each occasion, except that just preceding each subsequent infusion all the fluid which could be aspirated was removed and a sample inoculated into the various media. Cultures were observed for 10 days unless they became positive earlier.

Three normal dogs (No. 1-3) received a total of 10-40 liters in a period of 11-16 days. In 2, very little of the infused fluid was recovered by aspiration, but in the third, 30 of the 40 liters given were recovered.

Eighteen dogs were made uremic by removal of both kidneys. They are divided into 3 groups. The first group of 11 (No. 4-14) was treated in the same way as the normal dogs, but the food and fluid offered postoperatively was not consumed. In the second group of 3 (No. 15-17) a sterile foreign body (a rubber or stainless steel tube or both) was placed in the peritoneal cavity at the time of nephrectomy. In the third group of 4 (No. 18-21) 0.10 g of streptomycin and 1.0 g of sulfathalidine were given orally for 4-5 days prior to nephrectomy. Daily aerobic and anaerobic cultures of the stool were prepared and observed for several days preceding nephrectomy.

Blood was taken for culture from all dogs every second day until death.

Results (Table I) *Normal Dogs.* In all 3 normal dogs a few leucocytes per high power field appeared in the peritoneal fluid for the first time on the third day of the infusions. They increased only slightly in number thereafter. The peritoneum at the end of the experimental period was smooth, glossy, and entirely free of any reaction. No growth was obtained in any of the peritoneal fluid cultures. It is, therefore, evident that exposure of the peritoneal cavity of a normal dog to these irrigating fluids for much of each day for a period of some 2 weeks does not produce detectable bacterial contamination.

In 10 rabbits treated and observed in the same way for 14-16 days, the results were precisely the same.

Uremic Dogs. Untreated dogs survive not more than 3-4 days after bilateral nephrectomy. Two of the 11 dogs in Group I (No. 4-14) died on the 3rd and 4th day of infusion respectively. The aspirated fluid remained sterile. In the remaining 9, the aspirated fluid was sterile for the first 3 days of infusion. Leucocytes in the aspirated fluid appeared for the first time on the 2nd or 3rd day, and increased only slightly in number thereafter. Positive cultures were obtained for the first time from the fourth to the ninth day of infusion, and remained positive in all 9 dogs until death, which occurred 5-16 days after the infusions were started. *E. coli* was the contaminating organism in all instances; in pure culture in 2, together with *Aerobacter aerogenes* in 7. The number of colonies was always small, with *E. coli* predominating, except in Dog. 11. No clostridia were found in any of the anaerobic cultures. Only 2 dogs (No. 11 and No. 13) developed purulent peritonitis. In Dog 11, *E. coli, A. aerogenes* and *Pseudomonas aeruginosa* (*B. pyocyaneus*) were isolated. In Dog 13, *E. coli* and *Bacillus megatherium* were isolated.

In Group II (Dogs 15-17), contamination appeared for the first time on the 8th day of infusion in Dog 15, and on the 3rd day in Dog 17. Peritonitis did not develop in these dogs. Dog 16, which died of pneumonia 72 hours after nephrectomy, showed a sterile peritoneal inflammation after 48 hours of infusion. It appears that the sterile foreign body does not influence the time or degree of invasion of the peritoneal cavity by intestinal organisms.

In Group III (Dogs 18-21), the standard stool cultures remained sterile. A fecal suspension three times as dense as the standard yielded scanty growth of *Streptococcus fecalis* and diphtheroids, but no *E. coli* or clostridia. None of the four dogs in this group showed bacterial contamination of the aspirated fluid at any time, although the leucocyte response was the same as in the other

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|| Anaerobic cultures were done in 6 of the uremic dogs.

nephrectomized dogs. Their survival times were 5, 6, 9 and 12 days after the start of the infusions.

Death was due to uremia in all dogs in all 3 groups (except Dog 16) with purulent peritonitis contributing in Dogs 11 & 13.

The blood cultures were uniformly sterile. Cultures from the skin of the abdomen and of the irrigating fluid were always sterile before the infusion. Exceptionally a scanty growth was obtained from the skin after the infusion was given. The organisms recovered were the usual skin inhabitants, *i.e. Staphylococcus albus, Bacillus subtilis,* diphtheroids or sarcinae, and not the intestinal flora recovered from the aspirated fluid (Table I).

Discussion. The bacteria recovered from the aspirated fluid are clearly of intestinal origin, 1, because of the species found and 2, because their elimination from the intestine by oral chemotherapy prevents peritoneal contamination. The route of invasion is transmural, since the blood cultures were negative. The number of bacteria recovered in cultures was always small. Presumably this number is a measure of the balance between the number invading and the number leaving or being destroyed. It has been observed[3] that the peritoneum of dogs can destroy or eliminate a large number of bacteria without reacting with severe inflammation. Since the peritoneal cultures were uniformly negative in normal irrigated dogs, it appears that the uremic state makes the intestinal wall more permeable to bacteria or reduces the ability of the peritoneum to destroy them. The fact that only two of the fourteen uremic dogs which showed invasion developed purulent peritonitis may be due to the small number of invading bacteria or to the considerable natural resistance of the dog to these organisms.

It is noteworthy that the organisms recovered from the uremic dogs were the same as those from uremic patients treated with the same irrigating fluid.[1] Since the leucocyte response to the fluid in the normal animal is very slight, the fluid is at most a very mild stimulus to inflammation. Since neither the irrigating fluid alone nor uremia alone[1] is capable of producing peritoneal contamination, it appears that both factors acting together are responsible for the bacterial contamination. The precise role played by the uremic state is not clear. The fact that many of the uremic dogs showed an edematous intestinal wall at autopsy suggests that transmural migration may be due to the edema resulting from the absence of renal excretion, and that the uremia itself, so far as this phenomenon is concerned, is merely coincidental. However, in our clinical experience, the incidence of peritoneal contamination in irrigated patients was no less in those in whom overhydration was avoided. The ready development of *E. coli* peritonitis in man following contamination, as compared to animals, may be due to a lower natural resistance to *E. coli,* which the uremic state may further reduce.

This experimental study suggests that the treatment of acute renal failure in man by peritoneal irrigation may prove less hazardous, if a sufficient reduction of intestinal bacteria can be accomplished before treatment and maintained during treatment.

Summary and conclusions. Repeated intraperitoneal infusion of the fluid used for peritoneal irrigation in uremic patients, administered aseptically to healthy rabbits and dogs for 11-16 days, produced a moderate leukocytic response, but the peritoneum remained free of bacteria or visible inflammatory reaction. In uremic dogs, however, bacterial contamination of the peritoneal fluid developed after the 4th day of irrigation. The micro-organisms found were *E. coli* alone or with *A. aerogenes.* Occasionally, several other species, normally found in the intestine, appeared; but clostridia were never found. Preliminary oral administration of streptomycin and sulfathalidine prevented contamination of the peritoneal fluid. Blood cultures were uniformly sterile throughout the period of observation. It is concluded that irrigation of the peritoneal cavity of uremic dogs causes transmural migration of intestinal bacteria and that oral chemotherapy can prevent such contamination.

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[3] Steinberg, B., Goldblatt, H., *Arch. Int. Med.,* 1927, **39**, 446; 1927, **39**, 449.

17115. Experimental Studies on Nutrition in Tuberculosis. The Role of Protein in Resistance to Tuberculosis.[*][†]

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The view is widely prevalent, and well supported by demographic evidence, that a high level of dietary protein is protective against invasion by tuberculosis.[1-3] Recent experimental and other evidence has shown that protein metabolism is of basic importance in natural and acquired resistance to infection in general,[4] and shown that it may be of importance in tuberculosis.[5] The investigation here recorded was carried out to determine if a difference in susceptibility to tuberculosis can be detected in experimental animals nourished on diets of variable protein content.

In the stock of albino rats at the Wistar Institute of Anatomy and Biology, at the time these experiments were initiated, there were 3 colonies of animals which had lived for a number of generations on diets containing respectively 15, 25 and 40% of protein. Metabolic and growth studies on these animals have been reported by McCoy,[6] who found that rats raised on a diet containing 40% protein grew more rapidly and were more prolific than rats on diets with smaller protein content. Appearance and behavior differences were striking. The animals on a diet of 15% protein were sluggish and sleepy, while those on the 40% diet were active and playful. The former had ruffled and the latter sleek and shining hair. The 25% animals were between the two extremes.

The base diet furnished these animals was composed as follows:

	g
Whole wheat flour	3675
Skimmed milk powder	800
Whole milk powder	1200
Soy bean meal	400
Alfalfa leaf meal	300
Irradiated yeast	300
Dried liver	200
Calcium carbonate	25
Bone ash	80
Sodium chloride, iodized	20
	7000

From this base, 15, 25, and 40% diets were prepared as follows:

Composition of Diet, and Protein Level in Per Cent.

	15	25	40
Base	4200 g	4200 g	4200 g
Cod liver oil	240	240	240
Dextrin	1560	960	—
Casein	—	600	1560
	6000	6000	6000

Animals from the 3 groups were used for the experiments here recorded. Approximate equalization was effected within each protein group of experimental animals as respects age, weight and sex. All animals were injected intravenously with virulent tubercle bacilli of bovine type. Two separate experiments were carried out. The dosage was more accurately calibrated in the second than the first. In the first, animals of approximately equal weight were selected at the start and each received 0.1 mg tubercle bacilli in the internal jugular vein. In the second experiment, the injection carried out by the same route was so regulated that each animal received approximately the same dosage per unit lung weight. Lung weights were esti-

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[*] Aided by a grant from the John and Mary R. Markle Foundation.

[†] A preliminary report was presented at the IV International Congress for Microbiology, Copenhagen, Denmark, July 20-26, 1947.

[1] Faber, K., Tuberculosis and Nutrition, *Acta Tuberc. Scandinav.*, 1938, **12**, 287.

[2] Kirschner, M., *Z. f. Tuberk.*, 1921, **34**, 228.

[3] Schröder, G., *Beitr. z. Klin. d. Tuberk.*, 1930, **75**, 61.

[4] Cannon, P. R., *J. Am. Med. Assn.*, 1945, **128**, 360; Some Pathologic Consequences of Protein and Amino Acid Deficiencies, Charles C. Thomas, Springfield, Ill., 1948.

[5] Ratcliffe, H. L., *Am. Rev. Tuberc.*, 1946, **54**, 389.

[6] McCoy, R. H., *J. Biol. Chem.*, 1940, **133**, lxiv.

TABLE I.
Tuberculosis in Rats on Diets with Varying Percentage of Protein (Exp. 1).

% protein	Days of life	No. animals	Extent tuber.	Predom. type cellular react.	Avg degree localization	Avg No. bacilli	% with pneumonia
15	78-150	10	2.4	Epithelioid	Poor	Large	30
	167	2	3.0	"	"	"	100
25	47-150	9	2.7	"	"	"	44
40	78-150	5	2.4	"	"	Moderate	40
	196-293	7	2.4	Regr. mono.	"	"	100

TABLE II.
Tuberculosis in Rats on Diets with Varying Percentage of Protein (Exp. 2).

% protein	Days of life	No. animals	Extent tuber.	Predom. type cellular react.	Avg degree localization	Avg No. bacilli	% with pneumonia
15	14-150	10	2.1	Prog. mono.	Poor	Small	60
	180-222	4	1.8	Epithelioid	Moderate	"	25
	253-315	4	2.5	"	Poor	Moderate	50
25	14-150	4	1.5	"	"	"	50
	180-222	1	2.0	Regr. mono.	"	"	0
40	14-150	8	1.4	Prog. mono.	Good	Small	25
	180-222	6	1.5	Epithelioid	"	"	50
	253-315	10	1.3	Regr. mono.	Fair	"	20

mated from a formula worked out at the Wistar Institute.[7]

Animals were autopsied as they died or were killed at selected intervals. Sections were stained by hematoxylin and eosin, and also with carbol-fuchsin, by the technic for acid-fastness, in order to show the number of tubercle bacilli in tuberculous lesions. The most significant facts learned were with reference to pulmonary lesions, and the following analysis is based on the development of tuberculosis in the lungs.

A simple scoring system was adopted, which was based on (a) the extent of the lesion, (b) the type of cellular reaction, (c) the degree of localization of the tuberculous process and (d) the average number of tubercle bacilli present in lesions. In addition, because of what proved a significant development in the experiment, notation was made as to the presence or absence of extensive pneumonic lesions at the time of death. The extent of tuberculosis was estimated on a simple basis as follows: 1 = slight; 2 = moderate; and 3 = extensive.

The results of the 2 experiments are indicated in Tables I and II.

Table I illustrates the results of the first experiment, in which 33 rats, matched according to age and litter, were used. This group of animals developed pneumonia in a high percentage of cases. All of the animals on 15 and 25% protein, including those sacrificed, died within less than 168 days. Animals were sacrificed for comparable observation on the 78th and 150th day of the experiment. Eight rats on the diet containing 40% protein outlived all of the other animals, although all but one of these died from natural causes ultimately. The last animal of the group was killed on the 293rd day.

Tuberculosis developed rapidly in all animals and the extent exceeded "moderate" in all within the first 150 days. In the animals on the 15% protein diet very extensive tuberculosis occurred after the 150th day. After the 150th day in the animals on 40% protein little or no progression occurred, and in most animals a certain amount of regression developed, evident both by decrease in the size and extent of the lesion and by a tendency to regression in the type of reaction.

In general in the earliest days of infection a type of cellular reaction was evident which is here designated as a progressive mononuclear response. It was characterized by the early appearance of large mononuclear cells of the macrophage series with large nuclei and a small amount of cytoplasm. With the passage of time these mononuclear cells phagocytized tubercle bacilli and developed a large amount of cytoplasm, taking on the appearance of large epithelioid cells (epithelioid stage). The average type of reaction in all the animals up to the 150th day of the experiment was epithelioid. The 15 and 25% animals did not live long enough to develop the characteristic regressive type of mononuclear reaction. This was conspicuous, however, in the 40% animals, which lived much longer. This type of reaction was seen in most animals living longer than 180 days, and was particularly frequent in the animals receiving the highest concentration of protein. In this stage large mononuclear cells with a small amount of cytoplasm again became predominant and were present in far larger numbers than the typical epithelioid cells. Two explanations are possible for this phenomenon, (1) that the epithelioid cells gradually destroyed tubercle bacilli, after which the cytoplasm shrank, and (2) that epithelioid cells containing large numbers of tubercle bacilli were themselves destroyed and autolyzed, and replaced by new infiltrating large mononuclear cells, which found relatively few bacilli to phagocytize, and therefore did not develop the large amount of cytoplasm characteristic of epithelioid cells.

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[7] Donaldson, H. H., The Rat, Memoirs of the Wistar Institute of Anatomy and Biology, No. 6, 1924, p. 212.

In the animals on diets containing 15 and 25% protein tubercle bacilli were present in enormous numbers. In the 40% animals the number of bacilli present was definitely smaller.

In the 3 groups of animals in Experiment I, nourished on varying amounts of protein, significant differences were not evident in the incidence of pneumonia. In the first 150 days from 30 to 40% of all animals developed this disease and died. The few remaining 15% animals died of pneumonia at 167 days. The 40% animals lived many months longer, but all but the one finally killed ultimately succumbed to pneumonia.

Characteristic differences occurred in the weight curves of the 3 groups of animals. These curves are not plotted in this paper, because of the complication introduced by the fact that certain animals were killed, and the fact that the weights of others were irregularly influenced by the development of pneumonia. The general characteristics of the curves, however, were as follows: Animals on the 15% protein diet increased in weight from an average of approximately 190 g at the beginning of the experiment to approximately 280 g at about the 90th day, and decreased to approximately 220 g at the 150th day. Animals of the 25% series increased from an original average weight of approximately 200 g to an average of about 300 g on the 90th day and dropped to an average of 255 g on the 133rd day. Two surviving animals weighed approximately 310 g when killed at 150 days. Animals of the 40% series increased from an average of approximately 210 g at the start to about 300 g at the 84th day, and held this until the end of the experiment.

In summary it may be said of the animals of Experiment I that the extent of tuberculosis did not vary greatly in the 3 groups. However, the animals on the 40% protein diet outlived those on diets containing lower percentages of protein, by several months, and the number of tubercle bacilli visible in the lesions was much smaller than in the animals on 15 and 25% protein diets. At times in the 15% animals the number of bacilli was enormous, and wide dissemination of bacilli was evident in some of the animals dying of pneumonia. In a number of the animals with pneumonia ulceration of bronchi by the pneumonic process and wide scattering of exudate appeared to have a definite effect in spreading tubercle bacilli.

In Table II the results of the second experiment are recorded. Forty-seven rats were used. As noted above, the dosage was accurately measured on the basis of lung weight. The extent of tuberculosis in all groups was less than that in the preceding experiment, and differences in degree of localization were more clearly evident.

In the tabulation of Experiment II the animals are divided into 3 groups, *viz.*, those dying between 14 and 150 days, those dying between 180 and 222 days, and those dying between 250 and 315 days.

The extent of tuberculosis was measured on the same scale as in the case of the animals recorded in Table I. The extent of tuberculosis was somewhat greater in the animals receiving 15 and 25% protein diets than in those on the 40% diet. Also, as in Experiment I, after the first 150 days significant increases in extent of tuberculosis did not develop in the 40% animals.

In general the animals of this experiment showed the characteristic change in cellular reaction noted in Experiment I. This was most typical in the 40% animals, which clearly exhibited a progressive mononuclear reaction up to 150 days as the predominant type of reaction, followed by a long period during which the overwhelming majority of cells present in the exudate were of the epithelioid type, with a final period, after 250 days, when the epithelioid cells diminished in number, and were replaced by large mononuclear cells with relatively little cytoplasm. The latter stage, as noted above, is designated in this paper as regressive mononuclear stage.

In this experiment, in contrast to the first, distinct variations in the degree of localization of the tuberculous lesions were noted. In general the disease was widely disseminated in the 15 and 25% protein animals, but a "fair" to "good" degree of localization was evident in animals on the 40% protein diet. The number of bacilli visible in the tuberculous lesions was smaller in this experiment than in the previous one, but, as in that experiment, the number seen in tuberculous lesions was definitely smaller in the 40% animals than in those receiving smaller amounts of protein. Highly suggestive differences in the extent of pneumonia in the animals of the three groups were observed. The incidence of pneumonia was very high in the 15% group, and, while still high in the 40% group, it was much below that found in the 15% group. The difference in frequency and extent of pneumonia was more closely correlated with the amount of protein in the diet than was the extent of tuberculosis. The impression was created that, if the diet was responsible for variation in tuberculosis, the influence was indirect, through an effect upon resistance to the common types of pneumonia endemic in rat colonies. Bacteriological studies of the pneumonic lesions were not made. Previous studies on rats of this colony showed pneumonia is frequent and the etiology variable.[8]

As in the preceding experiment, in general the 40% animals far outlived animals of the other groups. At 315 days 5 of the 40% animals which had survived, were killed to conclude the experiment. These were large animals, free from pneumonia, with one exception, with excellent coats of hair, and every appearance of good health. As noted, all of them had moderately extensive tuberculosis, but this appeared to be in the regressive stage, as indicated by the type of cellular reaction.

Weight curves were comparable to those of the preceding experiment. In Experiment II, young and old animals were used, evenly distributed within the 3 groups. Young litters ranged from 61 to 76 days of age at the time of injection, and old litters from 736 to 931 days. All of the old animals died within the first 146 days. The animals which survived 315 days, all of them within the 40% group, were from 61 to 76 day litters. Differences in the type of cellular reaction were not observed between young and old animals.

Summary. The results of Experiment II were like those of Experiment I, the chief difference being that the infection was better regulated, and variations within the 3 protein groups were more characteristic. Tuberculosis was less extensive and life was much longer in the rats on a 40% protein diet than in the other 2 groups, and the degree of localization of the disease was better. The number of bacilli in lesions was smaller in the 40% series and the extent of pneumonia was less. Corresponding with the lesser extent of pneumonia there was less dissemination of tuberculous lesions. A characteristic feature of the long-lived animals was a progressive change in the cellular type of reaction from an early infiltration of mononuclears with little cytoplasm to a stage of consolidation with epithelioid cells and finally to a "regressive" type of mononuclear reaction in which lesions decreased in size, epithelioid cells were few and mononuclears with little cytoplasm predominated.

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[8] Ratcliffe, H. L., Chapter 22 in *The Rat in Laboratory Investigation*, Griffith, J. Q., and Farris, E. J., Lippincott Company, Philadelphia, 1942.

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17116. Ante-Mortem Failure of the Aural Microphonic in the Guinea Pig.*

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(Introduced by H. Lester White.)

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In the course of an extended study of the electrical response of the cochlea of the guinea pig to pure tones and to clicks we have collected incidental observations on the conditions of survival of these responses. Since these conditions are pertinent to any theory of the origin of the aural microphonic or "cochlear response" we present them herewith. Our report concerns only the aural microphonic, which apparently is closely related to the physical movements of the basilar membrane or some closely adjacent structure, and not the action potentials of the auditory nerve (or spiral ganglion) which are usually recorded with the microphonic. In general the action potentials fail equally with or before the microphonic. We have never yet observed the action potentials under conditions where the aural microphonic could not also be elicited, although sometimes a greater intensity of stimulus is necessary to make the microphonic visible on the oscilloscope.

Our sound-generating system and our pickup amplifiers and oscilloscopes follow conventional design and will be described in detail elsewhere. We make electrical contact with the cochlea through a tiny wick applied to the round window and through fine copper or silver wires passing through or fitted like a cork into small holes drilled through the bony shell of the cochlea. A reference electrode is connected to the edge of the wound in the neck.

Mere placement of an electrode on the round window membrane does not necessarily cause a reduction of the aural microphonic over a period of many hours or even 2 or 3 days. Neither does the anaesthetic *per se* (dial in urethane), provided the dose is light enough to allow reflex muscular movements and circulation and respiration remain adequate. Fall in body temperature sometimes seems to cause a reversible reduction of the microphonic, but no more than can reasonably be explained by the associated fall in metabolic rate and changes in circulation and respiration. Such effects of temperature have been more prominent in our experiments on cats than in guinea pigs.

It is well known that the aural microphonic falls off sharply when the animal dies, but that it persists for many hours after death at a few per cent of its original strength.[1] In the guinea pig, when respiration gradually fails from too much anesthetic, the microphonic falls to this low level *before* the heart stops. The failure is partly reversible. If the animal takes a few gasping breaths the microphonic may double its voltage (and action potentials return) as the heart beats faster and more regu-

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* This work was carried out under Contract N6onr-272 between the Office of Naval Research and Central Institute for the Deaf.

† Now in Santiago, Chile.

‡ Traveling Fellow of Rockefeller Foundation.

[1] Wever, E. G., Bray, C. W., and Lawrence, M., *Ann. Otol., Rhinol., and Laryngol.*, 1941, **50**, 317.

TABLE I.
Changes in Threshold (Decibels) for 20 Microvolt Response at Round Window (1000 Cycles) following Deliberate Injury.

Exp. No.	Injury	Time after injury 1 hr	2 hr	3 hr	21 hr
60	Turn 3; all 3 scalae	+ 4	− 3	—	− 7
63	,, 3; ,, ,, ,,	+14	+13	—	—
66	,, 2; large hole in scala media	0	—	—	—
67	,, 3; all 3 scalae, hole enters the modiolus	− 2		—	—
68	,, 3; amputation	+ 2	+ 4	+12	—
70	,, 3; amputation	− 4	− 4	—	—

Positive values indicate rise in threshold, negative a fall.
See Fig. 1A for Experiment No. 60, 1B for No. 67.

larly. It is our clear impression that there are two mechanisms underlying the microphonic, one of which is quite sensitive to lack of oxygen and the other sufficiently hardy to persist (at a low level) for hours after death.

Frequently, but not always, as respiration and circulation fail and as the microphonic approaches the postmortem level, the microphonic undergoes *half-wave rectification.* The phase of the microphonic in which the scala media and scala vestibuli are relatively more positive and the scala tympani more negative is depressed much more than the opposite half of the pattern. The phase which is most depressed corresponds to *condensation* in the external canal. This effect is most dramatic on the oscilloscope when clicks are used as stimuli, because the quiet base-line before the click gives an obvious reference level; but rectification can be clearly seen with pure tones also. The rectification is almost instantly reversible if the animal takes a few good breaths. At this stage rather strong stimuli, 30 or 40 db stronger than are necessary for a fresh preparation, are required to obtain clear responses and the maximum responses are rarely more than 10 microvolts. Action potentials always fail when or before rectification begins.

There are several sufficient conditions for failure of the microphonic, including lack of oxygen, overstimulation, certain chemicals applied to the round window (including the orthophosphoric acid we use in mixing the dental cement that attaches our electrode wires to the edge of the surgical opening into the bulla.) Gross injury, such as rupture of the round window membrane with outpouring of perilymph and perhaps entrance of air bubbles, also abolishes all responses.

Milder surgical injury associated with drilling a hole 300 μ in diameter and deliberately rupturing Reissner's membrane and the basilar membrane does not necessarily cause any deterioration of electric response over several hours except for a narrow band of frequencies associated with the actual site of injury. (See Table and Figure). In two experiments complete amputation of the 3d and 4th turns caused very little change in two hours of the stimulus necessary to yield at the round window a 20 microvolt response to medium and high tones. Small holes (60 μ) at the apex without rupture of the endosteum seem quite innocuous. We have not detected any effects that we must attribute to a moderate "escape of endolymph" or to "serous labyrinthitis."

Two general conditions are important, and they may reduce ultimately to one. They are 1) a general factor associated with progressive deterioration of the preparation in a long experiment. The electrical responses may gradually fall and thresholds rise. High tone responses are usually more depressed than low. 2) Local oxygen supply, which may be reduced by failure of circulation or by inadequate respiration. For example, the responses fail within a few seconds of cessation of respiration under pure nitrous oxide and return quite or nearly to normal promptly when breathing is resumed. The general factor suggested above may be nothing more than cumulative irreversible or slowly reversible effects of prolonged partial anoxia.

The stability of the responses in the presence of holes drilled into the cochlea opens the way for more intimate approach to the

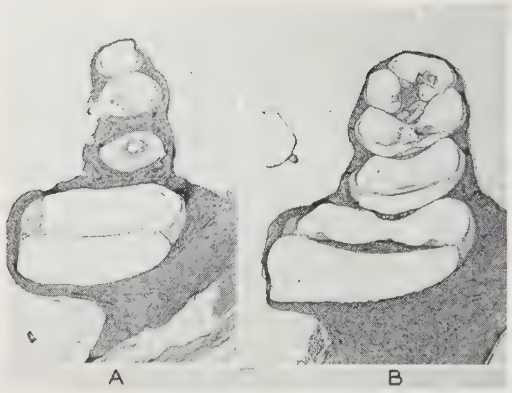

FIG. 1.

A. Exp. 60. Large hole and deliberate injury in Turn 3. Reissner's membrane and basilar membrane torn. Beneath the large hole is the outer end of a small hole that entered Turn 2, scala media.

B. Exp. 67. Hole entering modiolus in Turn 3. Axis of hole is perpendicular to plane of section.

nervous structures in labyrinth and modiolus.

Summary. The aural microphonic of guinea pigs was recorded from the round window and from copper wires applied to or passing through tiny holes drilled through the bony shell of the cochlea. When the guinea pig's respiration fails gradually, the microphonic falls to a few percent of its original strength before the heart stops. The failure is partly reversible if respiration improves.

Frequently near the postmortem level the microphonic undergoes partial or complete half-wave rectification. The phase corresponding to condensation in the external canal is more depressed than the phase corresponding to rarefaction. This effect also is temporarily reversible.

Extensive surgical injury including amputation of the two apical turns may cause little or no change in the microphonic (1000 cycles per second) at the round window over several hours.

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17117. Differences in Hemagglutination by Strains of Influenza Virus in Presence of Egg White and Normal Serum.*

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Substances of various origin have been shown[1-10] to inhibit the hemagglutination reaction of influenza virus. Evidence has also been presented[5,11,12] suggesting that some of these inhibitors are identical with, or closely related to, the receptor substance of red cells. When it was shown[13] that serum inhibits hemagglutination by heated virus to a much higher degree than hemagglutination by fresh

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[*] This investigation was conducted under the auspices of the Commission on Influenza, Army Epidemiology Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

[†] Aided by a fellowship from the Dazian Foundation for Medical Research.

[1] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

[2] Hirst, G. K., *J. Exp. Med.*, 1943, **78**, 99.

[3] Burnet, F. M., *Austral. J. Sc.*, 1947, **10**, 21.

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[6] Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 312, 442.

[7] Bovarnick, M., and deBurgh, P. M., *J. Exp. Med.*, 1948, **87**, 1.

[8] Ginsberg, H. S., Goebel, W. F., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, **87**, 411.

[9] Hardy, P. H., Jr., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, **88**, 63.

[10] Francis, T., Jr., and Minuse, E., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 291.

[11] Burnet, F. M., McCrea, J. F., and Anderson, S. G., *Nature*, 1947, **160**, 404.

TABLE I.
Comparison of Inhibition Titer of Two Samples of Rabbit Serum and Egg White on Hemagglutination by PR8 and Lee Strains.

Strain	Treatment	Inhibition titer* of			
		Rabbit serum No. 1	Rabbit serum No. 2	Egg white No. 1	Egg white No. 2
PR8	unheated	<10	<10	10	<10
	30′ 56°	<10	<10	40	<10
Lee	unheated	40	40	10	<10
	30′ 56°	160	160	2560	640

* Numbers give highest dilutions of inhibitor which are effective when added to virus.

virus, it was suggested that[12,14] the inhibition phenomenon could be explained on the basis of an enzymatic reaction as had previously been proposed[1] for the action of virus on red cells. According to the enzymatic theory, heating inactivates an enzyme of the virus which in the fresh state is able to destroy the inhibitor. The question arises whether all inhibitors are of the same chemical nature or whether different substances are involved. In terms of the enzymatic theory a plurality of inhibitors might also necessitate the assumption of different enzymatic factors in the virus. To obtain some evidence which could be used for answering this question, the behavior of different influenza strains toward the inhibitors contained in normal rabbit serum and egg white was compared.

Materials and methods. Virus Preparation. Allantoic fluid from eggs infected with the different strains of virus was used either unheated or after heating for 30 minutes at 56° or 61°C, during the first 14 days after harvesting. The following strains were examined: Swine, Oti, PR8, Weiss, Marton, Baum, FMl, Lee and Warner. The passage history of these strains is given in Table II.

Inhibitors. (a) Rabbit serum. Blood was drawn from normal, fully grown rabbits by cardiac puncture. After clotting and centrifugation, the serum (NRS) was pipetted off and heated for 30 minutes at 56°C.

(b) Egg white. The egg white (EW) of several eggs was separated from the yolk and filtered twice through a single layer of gauze. All fluids were stored at +4°C in rubber stoppered test tubes.

Inhibition tests. A titration of virus using Salk's method[15] was made before every test. A virus concentration equal to eight times the highest hemagglutinating dilution was used in the inhibition test. Two-fold dilutions of inhibitor in saline starting with a 10-fold dilution were prepared in 0.25 cc amounts, and to all dilutions 0.25 cc of the indicated dilution of virus was added. After thorough mixing the tubes stood at room temperature for 30 minutes. Then 0.5 cc of 0.25 per cent suspension of chicken blood cells was added. Results were read after 75 minutes. A parallel titration of the virus dilution used in the inhibition test was made in every experiment to insure that the right amount of virus had been used.

Results. Comparison of different batches of inhibitors. In a preliminary experiment the inhibiting action of 2 different batches of rabbit serum and egg white on the hemagglutination of PR8 and Lee strains was examined. It can be seen in Table I that the 2 rabbit sera had the same inhibition titer, but the 2 batches of egg white gave different results. The inhibition titers with Batch No. 1 of egg white were higher than with Batch No. 2. In comparing the effect of an inhibitor on different influenza strains, it is, therefore, important to use the same sample of inhibitor for the whole series. The experiments reported below were all done with the same samples of rabbit serum and egg white.

Susceptibility of unheated virus to NRS and EW. Table II shows that the examined

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[12] Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 301, 315.

[13] Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.

[14] Burnet, F. M., *Lancet*, 1948, **254**, 7.

[15] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

TABLE II.
Inhibiting Action of Rabbit's Serum and Egg White on Hemagglutination of Nine Different Strains of Influenza Virus.

Strain	Passage	Treatment	Inhibition titer[†] of rabbit serum	Inhibition titer of egg white
Swine	1976 M38 E25[*]	—	<10	10
		30′ 56°	<10	2560
Oti	M179 E37	—	10	40
		30′ 56°	40	5120
PR8	198 M593 E86	—	<10	20
		30′ 56°	<10	80
Weiss	F3 M32 E41	—	10	40
		30′ 56°	20	5120
Marton	E10	—	160	40
		30′ 56°	320	5120
Baum	F19 M10 E15	—	40	160
		30′ 56°	1280	5120
FM1	Lilly M37 E17	—	40	80
		30′ 56°	2560	5120
Lee	F8 M137 E122	—	40	10
		30′ 56°	160	2560
Warner	E6 M17 E2	—	640	20
		30′ 56°	2560	5120

[*] Number after F = number of ferret passages; number after M = number of mouse passages; number after E = number of egg passages.

[†] Numbers give highest dilutions of inhibitor which are effective when added to virus.

TABLE III.
Inhibition of Rabbit Serum on Influenza Strains Heated to 56°C and 61°C for 30 Min.

		Dilution of rabbit serum									
Strain	Treatment	10	20	40	80	160	320	640	1280	2560	5120
PR8	56°	+	+	+	+	+	+	+	+	+	+
	61°	—	—	—	—	—	—	—	+	+	+
Weiss	56°	—	—	+	+	+	+	+	+	+	+
	61°	—	—	—	—	—	—	—	—	+	+
Oti	56°	—	—	—	+	+	+	+	+	+	+
	61°	—	—	—	—	—	—	—	—	+	+
Lee	56°	—	—	—	—	—	+	+	+	+	+
	61°	—	—	—	—	—	—	—	+	+	+

+ = hemagglutination.
— = no hemagglutination.

strains differ in their behavior toward the 2 inhibitors. Six strains are more susceptible to inhibition by EW, three strains are more strongly inhibited by NRS. The greatest difference in behavior toward the 2 inhibitors was observed in the case of the Warner strain which was inhibited in a 32-fold higher dilution of NRS than of EW.

Susceptibility of heated virus to NRS and EW. Heating for 30 minutes at 56°C made all strains except PR8 highly susceptible to the inhibiting action of EW. Inhibition titers of 2560 to 5120 were obtained. The susceptibility to inhibitor after heating of the virus was one hundred to several hundred fold greater with all strains except PR8 with which the rise was only 4-fold. The effect of heating virus preparations to 56°C on the susceptibility to inhibition by serum was different. In 5 strains the rise in susceptibility was only 2 to 4-fold and in the case of PR8 and swine influenza strain 1976 this temperature had no measurable effect on the susceptibility to NRS. Especially striking was the behavior of the swine influenza strain. Its susceptibility to EW rose several hundred times, while its susceptibility to NRS was not changed. A similar observation with the same virus has already been made by Lanni and Beard.[6] With two strains the rise in titer of NRS was 30 to 60-fold and thus approached the values observed with EW.

With other strains the inhibition of hemagglutination by NRS could be raised to titers similar to those attained by EW if a temperature of 61°C was used for the inactivation of the virus preparations. This is shown in Table III for the PR8, Weiss, Oti and Lee strains. NRS in dilutions of 640-1280 inhibited hemagglutination by heated virus, and thus came close to the values obtained with EW. In the case of the swine influenza strain it was impossible to raise its susceptibility toward NRS as a temperature of 56°C was ineffective and a temperature of 58°C destroyed the hemagglutinating ability of this strain.

Discussion. The observation that an influenza strain may be highly susceptible to one inhibitor and only slightly susceptible to another and the fact that heating will alter the susceptibility of a strain to one inhibitor without changing measurably its susceptibility toward the other is considered evidence for a qualitative difference in the inhibitors involved. If this conclusion is discussed in terms of the enzymatic theory it would have to be assumed that the effect of virus on the two inhibitors is caused by two different enzymatic factors which can exist independently and which can be differentiated by their resistance to heat. The virus factor active against egg white inhibitor is generally more heat labile than the factor against rabbit serum. It is completely or partially destroyed in all strains by a temperature of 56°C whereas, a higher temperature is necessary to inactivate the factor acting against rabbit serum. The independence of the two factors is most clearly demonstrated in the case of the swine influenza strain in which heating to 56°C destroys completely the activity against egg white without measurably altering its activity against serum inhibitor. It is possible that the assumption of still other enzymatic factors for other inhibitors might become necessary, if the enzymatic theory is to be accepted. It seems probable that just as the antigenic composition of strains differs so also will their capacity to react with different chemical substrates be found to vary.

Summary. The hemagglutinating activity of some strains of influenza virus is more susceptible to egg white inhibitor while that of others is more susceptible to inhibition by rabbit serum. Heating to 56°C makes all strains susceptible to inhibition by egg white. When tested with 4 different strains a higher temperature was required to make them as susceptible to inhibition by normal rabbit serum. It is concluded from these results that the inhibitors are not identical. The results are discussed in terms of the enzymatic theory according to which two different enzymatic factors in the virus active against rabbit serum and egg white would have to be assumed.

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17118. Effect of Tagathen* on Histamine-Induced Gastric Lesions in Guinea Pigs.

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The use of synthetic antihistaminics to prevent or diminish the incidence or severity of histamine-induced ulcers in animals has been reported in less than a half dozen papers. Most of these were concerned with the failure of benadryl* (B-dimethylaminoethylbenzhydryl ether HCl) to prevent experimental ulcer formation.[1–4] In all of these reports the "histamine in beeswax" method of Wangensteen and coworkers[5] was used. In addition in a recent paper, the pyloric ligation method of Shay and coworkers[6] was used to evaluate the anti-ulcer effect of thephorin* (2-methyl-9-phenyl-2,3,4,9 tetrahydro-1-pyridindene).[7]

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* Brand of antihistaminic drug.

[1] Crane, J. T., Lindsay, S., and Dailey, M. E., *Am. J. Dig. Diseases*, 1947, **14**, 56.

[2] Friesen, S. R., Baronofsky, I. D., and Wangensteen, O. H., Proc. Soc. Exp. Biol. and Med., 1946, **63**, 23.

In our initial attempts to establish an assay for the investigation of the influence of diet on histamine-induced ulcer, we tried the histamine in beeswax method. In our hands it frequently resulted in the death of a considerable number of animals due to bronchial spasm. To prevent this the animals were pretreated with tagathen* (N,N-dimethyl-N′-(2-pyridyl)-N′-(5-chloro-2-thenyl) - ethylenediamine citrate) or with epinephrine. Under these conditions we were able to produce only an occasional gastric lesion in a large number of animals. After numerous trials it was found that gastric lesions could be consistently produced by the intramuscular injection of 0.6 mg histamine diphosphate in an aqueous solution twice daily. The number of deaths due to bronchial spasm was practically nil. This led to the study of the influence of diet and various drugs on histamine-induced ulcer in guinea pigs.

Method. Hartley strain guinea pigs weighing 200-350 g were divided into groups containing equal numbers of each sex and average weight. Animals were placed in individual cages and fed the appropriate diet for 5-7 days. At the end of this period the drug supplement was administered twice daily, morning and afternoon, one hour before the intramuscular injection of 0.6 mg histamine diphosphate in distilled water. Food was removed from cages before the first histamine injection and returned 2-3 hours after the second injection. This treatment was continued for 4½ days and all surviving animals were killed and autopsied. Animals succumbing before the end of the test were autopsied as soon as possible after death. Animals were examined for the presence or absence of gastric hemorrhages, erosions and/or perforation.

The synthetic diet used in this work has the following composition expressed in grams: Cerelose 500, Alcohol extracted casein 220, Cellulose 150, Gelatin 60, Salt mixture 50, Corn oil 20, Vitamin A 19,720 I.U., Vitamin D 2,360 I.U., Vitamin E 0.025, Vitamin K .0005, Riboflavin 0.005, Inositol 0.05, Thiamine HCl 0.003, Pyridoxine HCl 0.005, Calcium pantothenate 0.02, Nicotinamide 0.01, p-aminobenzoic acid 0.01, Choline Cl 1.0, Biotin 0.0001, Folic Acid 0.001.

The animals were given 20 mg ascorbic acid orally 3 times per week. This diet was also fed with kale *ad libitum*.

Results. The results are presented in Table I. A high incidence of histamine-induced lesions were obtained on both the basal diet, and the basal plus kale *ad libitum*. On the former the percentage was 92%; on the latter 98%. The omission or addition of extra ascorbic acid to the synthetic diet did not appreciably influence the effect of the histamine.

Since our earlier work indicated that tagathen administration interfered with the production of ulcers by the histamine in beeswax technic, it was decided to investigate the effect of this antihistaminic drug thoroughly. The data in Table I rather clearly demonstrate that the compound if given in sufficient dosage, will prevent a high percentage of the animals from developing gastric lesions. This is attested by the fact that out of 92 guinea pigs receiving 4 or more milligrams of this antihistaminic drug per dose, only 36 exhibited gastric lesions. A considerable number of those affected showed only minimal damage as compared to their controls. At higher dosages than 4 mg the percent incidence is further reduced. This, we believe to be remarkable in view of the previously reported inability of this type of anti-

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[3] Harkins, H. N., Hooker, D. H., Alford, T. C., Callander, J., Elliott, S. R., Kearns, W., Mitchner, J., and Cooley, D. A., *Bull. Johns Hopkins Hospital*, 1947, **81**, 79.

[4] Vallery-Radot, P., Halpern, B. N., and Martin, J., *Presse Med.*, 1947, **55**, 185.

[5] Hay, L. J., Varco, R. L., Code, C. F., and Wangensteen, O. H., *Surg., Gynec. and Obst.*, 1942, **75**, 170.

[6] Shay, H., Komarov, S. A., Fels, S. S., Merenze, D., Gruenstein, M., and Siplet, H., *Gastroenterology*, 1945, **5**, 43.

[7] Lehmann, G., and Stefko, P. L., *J. Lab. and Clin. Med.*, 1949, **34**, 372.

TABLE I.
Influence of Tagathen on Production of Histamine-Induced Ulcers in Guinea Pigs.

Treatment*	No. animals	Animals showing gastric pathology No.	%
Kale†	11	0	0
Kale† + histamine‡	45	44	98
Histamine‡	25	23	92
No Vit. C + histamine‡	7	5	71
20 mg Vit. C + histamine‡	6	5	83
4 ’’ tagathen§	6	0	0
4 ’’ ’’ + histamine‡	6	0	0
6 ’’ ’’ ’’ ’’	6	1	17
0.1 ’’ ’’ ’’ ’’ + kale†	6	6	100
0.5 ’’ ’’ ’’ ’’ ’’ ’’	6	6	100
1 ’’ ’’ ’’ ’’ ’’ ’’	18	10	56
2 ’’ ’’ ’’ ’’ ’’ ’’	12	7	58
4 ’’ ’’ ’’ ’’ ’’ ’’	50	24	48
6 ’’ ’’ ’’ ’’ ’’ ’’	19	9	47
8 ’’ ’’ ’’ ’’ ’’ ’’	6	1	17
12 ’’ ’’ ’’ ’’ ’’ ’’	5	1	20

* All groups received basal diet as given except as noted.
† Kale—fed *ad libitum.*
‡ Histamine diphosphate (0.6 mg) in distilled water injected i.m./pig 2×/day.
§ Tagathen in distilled water orally/pig 2×/day.

histaminic to prevent histamine-induced ulcers. It would appear that such a reduction in ulcer production in so large a group of animals clearly demonstrates the potent anti-ulcer properties of this compound.

In addition to the above reported experiments we have made preliminary investigations on the influence of desiccated thyroid, thiouracil, dried liver cake, 15-unit liver extract, insulin, thyroxine, tannic acid, 2,4-dinitrophenol and casein digests on the incidence of histamine-provoked gastric lesions.[8] None of these was found to have any marked preventive or provocative activity.

Summary. Conditions have been established for the production of gastric lesions in guinea pigs by injection of aqueous histamine without considerable loss of animals due to fatal bronchial spasm.

Tagathen causes a very pronounced reduction in the incidence of gastric lesions produced by this method.

In view of the favorable results obtained, it would seem that a study of the mechanism involved in the anti-ulcer effect produced by antihistaminic compounds of this type, should be made.

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[8] Van Meter, J. C., unpublished data.

17119 P. Blood Flow and Oxygen Consumption of the Brain in Coarctation of the Aorta.*

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Recent developments making possible measurements of the blood flow to the brain[1,2] and myocardium[3] in man[4] enable the clinical investigator of the hemodynamics in coarctation of the aorta to make for the first time an accurate estimate of the pressure-flow relationship in these regions. Reports concerning the circulatory alterations in this malformation have not established decisively whether or not vascular resistance is increased uniformly throughout the body.[5-10] Bing suggests that there is no generalized elevation of peripheral vascular resistance.[11] This preliminary report is to present evidence showing that cerebral blood flow and oxygen consumption in two patients with coarctation are significantly increased above normal.

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† Dr. Crumpton is National Health Institute Fellow.

Studies in this laboratory over the past four years have indicated that there is increased resistance to blood flow through the brain in arterial hypertension of unknown cause, this region sharing in equal measure the generalized increase in vascular tone which is associated with essential hypertension.[12,13] We

TABLE I.

Cerebral Circulatory Data with Normal Circulation,[2] Essential Hypertension, and Coarctation of the Aorta.

Group	Cerebral blood flow, cc/100 g/min.	Blood pressure, mm. Hg. Indirect: Brachial, Right, Obs.	Right, Mean	Left, Obs.	Left, Mean	Direct: Femoral mean	Cerebral vascular resistance MABP/CBF	Cerebral arteriovenous O$_2$ diff. vol. %	Cerebral oxygen consumption cc/100 g/min.
Young normal ♂ (34 observations, 14 subjects)	54	107/69	82			86	1.6	6.3	3.3
Coarctation group, mean	*78		*†115				1.5	6.0	*4.6
S.S. 18 WM	87	130/105	113	180/100	127	107	1.5	6.0	5.2
R.F. 20 ”	68	150/80	103			84	1.5	5.9	4.0
Essential hypertension (45 observations, 45 patients)	54					*156	*3.1	6.5	3.2

* Denotes statistically significant difference from normals.

† This is the average value of mean brachial artery pressure for the two coarctation patients where the higher brachial artery pressure of S.S. (127) was chosen as the one more closely approximating the value of mean carotid artery pressure.

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‡ The authors wish to acknowledge the assistance of Drs. C. F. Schmidt, Seymour S. Kety and William A. Jeffers in making these studies.

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[13] Kety, S. S., Hafkenschiel, J. H., Jeffers, W. A., Leopold, Irving H., and Shenkin, Henry A., *J. Clin. Invest.*, 1948, **27**, 511.

have had the opportunity to make measurements of the cerebral circulation in two patients with hypertension limited to the upper part of the body by reason of their having coarctation of the aorta. Our results indicate that the cerebral vascular resistance in these patients is lower than that found in essential hypertension.

Methods. Cerebral blood flow, cerebral oxygen consumption, mean femoral pressure and brachial artery auscultatory pressure measurements were made in two patients with coarctation and in forty-five with essential hypertension.[2] The mean pressure of the coarctation patients was calculated by using the diastolic pressure plus one third the pulse pressure as obtained by brachial artery auscultation.[14] The pressure in the carotid artery was assumed to be the same as the brachial artery pressure. The cerebral vascular resistance for the essential hypertension patients was calculated as previously described.[2]

Results. These are presented in Table I. Cerebral blood flow and calculated mean carotid pressure are significantly increased when compared with the mean values of the young normal male.[2] These data indicate that the cerebral vascular resistance in coarctation is normal, and is not increased as in essential hypertension. Cerebral oxygen consumption is also increased with cerebral arteriovenous oxygen difference not significantly different from the normals or hypertensives.

Discussion. Because S.S. had different blood pressures in each arm the higher pressure (127 mm Hg) is assumed to be more representative of the pressure in the cerebral arterial circuit. These pressure values probably underestimate mean carotid artery pressure by about 5 mm Hg because brachial artery auscultation is a lateral artery pressure measurement. This does not take the coarctation cerebral vascular resistance values out of the normal range.

Summary. 1. Measurements of cerebral blood flow, oxygen consumption and cerebral vascular resistance were made in two patients with coarctation of the aorta. The resistance to blood flow through the brain is found to be within the normal range.

2. This is in contrast with the increased cerebral vascular resistance of essential hypertension.

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[14] McLeod's Physiology in Modern Medicine, 8th edition, edited by Philip Bard, Chap. 21, p. 314.

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